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Space Bioreactor Science Workshop

Dennis R. Morrison, *Editor*
Lyndon B. Johnson Space Center
Houston, Texas

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PREFACE

GOALS

The intent of the Space Bioreactor Science Workshop was to consider the design and potential utilization of a stirred perfusion-type cell culture system being developed to culture mammalian cells under microgravity conditions. The workshop was held August 22-23, 1985, at the Nassau Bay Holiday Inn adjacent to the Lyndon B. Johnson Space Center in Houston, Texas. Eighteen well-established and recognized research scientists and engineers from industry and academia, selected on the basis of their expertise and accomplishments within their field, were invited to present papers. The topics of discussion were selected by the workshop organizers (D. R. Morrison, C. D. Anderson, and S. L. Pool) according to the relevance to the design or use of cell culture systems.

An important consideration in the selection of the invited speakers was that they have some familiarity with the basic concepts and design requirements for the space bioreactor. However, most were unaware of the special considerations required for proper operation in micro-g. As a result, invitees were supplied with background information weeks before the workshop in order to acquaint them with the space bioreactor project and its intended scientific use during the first flight experiments. It was felt that this approach would help in generating new ideas and perspectives. Pursuant to the presentations, the speaker's ideas were subsequently tempered through open discussions with an audience comprised of academic researchers, NASA scientists, representatives from NASA Headquarters, and the contractor teams who were developing this system. This arrangement fostered a lively exchange of ideas and a critical examination of each paper.

ORGANIZATION

The workshop was organized so that each panel member was allotted 30 minutes to present a prepared talk with a 10-15 minute discussion period afterward. Impromptu discussions continued during coffee breaks and lunch and during a general session at the end of the formal presentations. On the first day, NASA scientists and contractors (Technology Inc./currently Krug International) discussed the approach and design of the first space bioreactor. These presentations were followed by the delivery of chemical engineering papers on related issues. The second day included presentations on new sensor systems, oxygen requirements, nutrient utilization, and potential new applications for the culturing of nonmammalian cells in microgravity. At the invitation of the organizers, most of the workshop participants elected to submit formal papers which are included in these proceedings.

RESULTS

Based on questionnaires filled out by all participants, the meeting was considered quite successful and provided a forum for a good exchange of ideas and concepts. Most of the participants agreed that a bioreactor designed to culture mammalian cells on microcarriers would also be useful for the culture of other fragile cells which are difficult to culture in conventional fermentors or stirred bioreactors.

Major problem areas in perfusion culture technology were identified such as control of adverse shear, inadequate sensors, and the need for multi-tasking microprocessor controllers which are more sophisticated than PID controllers. The need for accurate, real-time measurement of cell metabolism was emphasized. Suggestions were made for strategies to assess the cellular response to shear and other stressors in perfusion bioreactors. Finally, some minor additions to the design requirements were suggested which could enable reactor vessel changeout to accommodate plant and yeast cell experiments on future space flights.

It was recommended that NASA conduct a similar workshop every 2 years as the space bioreactor hardware and flight experiments are developed.

FOREWORD

During the interval between the Space Bioreactor Science Workshop in August 1985 and the publication of these proceedings, a number of fluid mechanics and biochemical engineering studies have been conducted at several universities. Several tests and cell culture experiments also have been conducted at JSC laboratories. This foreword is intended to update the reader on the status of this bioreactor research and, in particular, to indicate what progress has been made along the lines of inquiry recommended at the workshop.

BIOREACTOR FLUID MECHANICS

1. Effects of Mechanical Stress in Suspension Bioreactors

Studies have been conducted on the mechanical stress imposed on cells as the result of various agitation methods used in stirred bioreactors. Analysis of the possible mechanisms of cell damage has shown that cell death in microcarrier cultures is largely a result of collisions between the microcarrier beads and the impeller and collisions with other beads (Cherry and Papoutsakis, 1986). The cells are most affected, however, by turbulent eddies which cause rotation and high local shear at the bead surface, thereby damaging the fragile cells attached to the beads. The cells are also exposed to 10-20 Hz cyclic shear stress from bead rotation in the flow fields.

Several runs have been performed on bovine kidney cells grown for as many as 14 days at agitator speeds of up to 100 rpm. Growth rates and viability were correlated with the degree of confluence and the amount of bead-to-bead cell bridging which can occur (unexpectedly) at high stir rates. Future studies will address the mechanisms by which excessive agitation reduces growth and viability of cells grown on microcarrier beads. The results of these studies should be applicable to suspension culture of hybridoma cells and other fragile anchorage independent cells.

2. Fluid Dynamic Modeling of Bioreactor Vessels

As new bioreactor designs are tested, more information is needed on hydrodynamic flows within the reactor vessel. Studies of flow patterns and measurement of point velocities within the stirred vessels have determined the shear environment under various operating conditions. Several measurement techniques have been developed for determining the actual shear levels imposed upon cells attached to microcarrier beads

in a suspension bioreactor. These include video recordings and analysis for overall flow patterns as well as laser Doppler velocimetry for point-velocity measurements. Local point-velocity measurements have now been completed for several vessel geometries, including low-aspect ratio vessels with flat and spiral vanes as well as a horizontal clinostat constructed at JSC (Nerem and Kleis, unpublished results). These methods also are being considered for use in the first space bioreactor flight tests to determine actual shear levels at very slow stirring rates at which microcarrier beads will not sediment.

CELL CULTURE STUDIES

1. Biophysical Effects of Fluid Shear

In 1985, flow chamber studies showed increased secretion of urokinase from primary cultures of human embryonic kidney cells subjected to low shear stress (Stathopoulos and Hellums, 1985). A similar result was found for the secretion of prostacyclins by cultured human endothelial cells (Frangos, et al, 1985). New studies using monolayer cultures in special laminar flow chamber are determining the optimum shear levels for minimum cell stress and maximum urokinase secretions (Morrison and Kalmaz, unpublished results). The results of these studies will be used to establish target ranges for low-level shear operations during flight experiments in the space bioreactor.

The stress response of cultured kidney cells subjected to elevated temperatures (42°C) is being compared with the response to short exposures of low-level fluid shear (12 dynes/cm²). Radioactive labeling (S-35 methionine) and 2-D gel electrophoresis have shown that intracellular synthesis of the 70,000 Da stress response proteins was greatly increased after low-level shear stress. Instead, the synthesis of approximately 12 other intracellular proteins was turned on as a result of exposure to this level of shear stress (Morrison and Goochee, unpublished results). The current objective is to develop a sensitive method for measuring the stress levels of cells cultured in stirred bioreactors. Results from studies in this area should have application to the first microgravity experiments with the space bioreactor in which shear levels can be maintained below those which are practical on Earth.

2. Cell Culture Experiments in the Prototype Bioreactor

In the JSC bioprocessing laboratories four major cell culture experiments have been conducted to test the operations of the bioreactor lab test units. Changes in reactor vessel design have been the major focus of this engineering effort. Major new designs have been tested to determine

how long human kidney or baby hamster cells can be grown and kept viable under the most optimum conditions in 1-g. The effects of stirring rates, oxygen supply, nutrient depletion, and other environmental parameters are being measured to determine whether or not the bioreactor designs are improvements over traditional laboratory systems. Comparisons are being made with similar microcarrier cultures, using three different commercial bioreactors, at the chemical engineering departments of the University of Houston and Rice University.

The current design of the lab test unit is a horizontal cylinder with stirring vanes set close to the wall. The unit operates without head-space to avoid the surface tension separation of gasses and the liquid culture medium that occurs in the absence of gravity. This particular design resulted from operational concepts which allowed for the fact that the Shuttle Orbiter lifts off vertically and lands horizontally. Using a horizontal axis of symmetry in the cylindrical vessel avoids changes in the orientation of the vessel when it is mounted in the X-axis of the Orbiter. In this system, flow visualization with guanine crystals has shown less turbulence in comparison with conventional stirred reactors (Kleis, unpublished results). Residence time distribution tests have shown that fluid mixing also is good in this vessel. Cell growth studies have been performed to determine seeding densities and attachment rates to microcarriers during constant stirring. BHK cells have been grown in high-serum medium for 2 weeks in the latest lab test unit (Lewis et al., 1986).

PROCESS CONTROL

A custom microprocessor controller has been developed based on a STD bus system and a multitasking basic software program has been developed and tested (Hall and Bowie, unpublished results). With contemplated improvements in hardware systems design, this controller and software should be adequate for the flight bioreactor. Process control strategies were reviewed by outside experts and recommendations were made to measure sensor lag, feedback reaction times, acid-base addition, and reactor vessel clearance times to properly design control algorithms (Lim, NASA communication). Some of these parameters have been quantified (Cross et al., 1986) and some work has been done using artificial intelligence for process control strategies (San and McIntire, Rice University, unpublished results).

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Dennis R. Morrison, Ph.D., editor

INTRODUCTION

In 1969, concepts were developed for a fermentation-type cell culture system designed to operate in microgravity. Until recently, however, cell culture experiments performed in space were confined to small (5-10 ml) growth or holding chambers in basic incubators. Experiments were necessarily small and simple, permitting only basic observations and some sample fixation for postflight analysis back to Earth. Since 1975, NASA has continued a small, but methodical research program to develop principles for more sophisticated cell culture and bioprocessing experiments. Cell culture science conferences at MIT, the Salk Institute, and the NASA/Johnson Space Center have identified many areas where cell culture experiments in microgravity may provide unique insight into the basic mechanisms of cell biology. These cell culture experiments may also offer an insight into the potential advantages of growing and maintaining fragile mammalian cells in a precision controlled environment under gentle conditions of low shear. For more than 8 years, industrial contract research and academic collaborations have been used to enhance JSC's in-house experience with conventional suspension culture systems and to develop novel designs required for operations in microgravity. The prototype of the first perfusion-type space bioreactor which will grow and maintain human cells on microcarriers is now being developed. Many diverse experiments have been proposed which could utilize this facility; therefore, design requirements have been specified to provide both generic capabilities and those special capabilities required by anchorage dependent human cells.

The intent of this Space Bioreactor Science Workshop was to consider the design and potential utilization of a stirred perfusion-type cell culture system being developed to culture mammalian cells under microgravity conditions. Those who participated in the workshop were well-established and recognized research scientists from industry and academia who were familiar with the NASA space bioreactor project and with recent innovations in human cell culture systems. It was envisioned that the participants could serve as an ad hoc review group to both critique the current efforts and to establish additional design considerations providing more versatility for future space bioreactor users. Most attendees were unaware of the special considerations required for proper operation of the space bioreactor in microgravity, but were provided with background information prior to the workshop in order to acquaint them with the space bioreactor project and its intended scientific use during the first flight experiments. It was felt that this approach would help generate new ideas and prospectives and as a result, many participants presented papers on novel cell culture systems and the potential use of the space bioreactor, with a minimum of modification, for culturing plant protoplasts, yeast, and other cells.

Most of the participants agreed that a bioreactor designed to culture mammalian cells on microcarriers would also be useful for the culture of other fragile cells such as human hybridomas, wall-less plant protoplasts, and other cells which are difficult to culture in conventional fermenters or stirred bioreactors. Major problem areas in perfusion culture technology, such as the control of adverse shear, inadequate sensors, and the need for multitasking microprocessor controllers which are more sophisticated than PID controllers, were identified. The need for accurate, real-time measurement of cell metabolism was emphasized. Suggestions were made for strategies to assess the cellular response to shear and other stressors in perfusion bioreactors. Finally, some minor additions to the design requirements were suggested which could enable reactor vessel changeout to accommodate plant and yeast cell experiments on future space flights. These papers have been published in this NASA document to provide a reference for design engineers and researchers who may wish to consider use of this space flight system for future experiments on the Space Shuttle. It was also recommended that NASA conduct a similar workshop every 2 years as the space bioreactor hardware and flight experiments are developed.

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SUSPENSION CELL CULTURE IN MICROGRAVITY AND DEVELOPMENT
OF A SPACE BIOREACTOR

Dennis R. Morrison, Ph.D.
Manager, Space Bioprocessing Laboratories
NASA Johnson Space Center
Houston, TX 77058

ABSTRACT

Microgravity offers new ways of handling fluids, gases, and growing mammalian cells in efficient suspension cultures. Some conceptual designs for a zero-g fermentor were developed as early as 1969. In 1976 bioreactor engineers designed another system using a cylindrical reactor vessel in which the cells and medium are slowly mixed. The reaction chamber is interchangeable and can be used for several types of cell cultures. NASA has methodically developed unique suspension type cell and recovery apparatus culture systems for bioprocess technology experiments and production of biological products in microgravity. The first space bioreactor has been designed for microprocessor control, no gaseous headspace, circulation and resupply of culture medium, and slow mixing in very low shear regimes. Various ground-based bioreactors are being used to test reactor vessel design, on-line sensors, effects of shear, nutrient supply, and waste removal from continuous culture of human cells attached to microcarriers. The small (500 ml) bioreactor is being constructed for flight experiments in the Shuttle middeck to verify systems operation under microgravity conditions and to measure the efficiencies of mass transport, gas transfer, oxygen consumption, and control of low shear stress on cells.

INTRODUCTION

For several decades, many important pharmaceuticals and other biological products have been obtained from large scale culture of microbial cells. However, the mass cultivation of mammalian cells is much more difficult because of their delicate nature and their stringent environment requirements.¹ In fact, contemporary culture technology is often inadequate in attempts to provide the proper conditions for optimum human cell growth and cell secretions. During the past 10 years the need for major technological improvements in human cell culture has increased because of the expanding need for human cell products, such as hormones, enzymes, interferon, etc., to be used as pharmaceuticals.

Increased demand for large numbers of cells for virus and cancer research has also added impetus to develop practical systems for large scale culture

of mammalian cells. Some improvements have been made; however, large scale culture systems still have major problems which make human cell culture very difficult and often uneconomical.²

A great deal of excitement has resulted from the recent success in the genetic reprogramming of bacterial cells to produce human cell products such as growth hormones, human insulin, and interferon. However, the genetic engineering of very complicated molecules is still several years from commercial practicality. Products are still contaminated with bacterial proteins and DNA fragments producing problems with allergenic or immune reactions to the preparation. To date only growth hormone, obtained from genetically engineered cells, has been approved by the Food and Drug Administration. Another limitation is the inability, so far, to genetically code for any glycosylated proteins. Certain human cell products which may not be practically produced by genetic engineering remain good candidates for production by culture of human cells whenever the technological problems are solved. Many diseases involving neoplasms, blood clots, hypertension, anemia, emphysema, growth disorders, and others are now considered treatable with pharmaceuticals derived from cell culture if only the technology can be improved to make large scale cultures practical.^{3,4}

In the NASA bioprocessing laboratories at the Johnson Space Center we have evaluated several of the conventional and more recent methods for culturing human cells. Our studies involved (1) the definition of the environmental conditions that must be controlled to ensure successful growth and maintenance of human cells under microgravity conditions; (2) the recovery of cultured cells and cell products for postflight research; (3) the initial isolation or first step in downstream processing which may be used as a feeder step to the purification of products by continuous flow electrophoresis (CFE) or recirculating isoelectric focusing (RIEF); and (4) the design features which must be included to maintain precise control of culture conditions under microgravity conditions.⁵

In order to design proper control systems, it was essential that we obtain performance data on various component elements of the culture apparatus. Special emphasis was placed on designs for fluid handling, temperature distribution, gas/liquid phase separation, etc., under microgravity conditions. We approached these problems by first identifying physical phenomena and operational principles of suspension culture which are gravity dependent. Then scientific workshops were held to determine which phenomena may be altered in microgravity to produce significant advantages or disadvantages in the culture process.^{6,7} It became apparent that many physical and biophysical interactions within cell culture systems are poorly understood. Several areas appeared to need additional research to better understand the importance of particular physical interactions which occur in suspension culture systems (see table 1-1).

EVALUATIONS OF EARTH-BASED CELL CULTURE SYSTEMS

Classic industrial and research methods of growing human cells have been limited to systems which grow and maintain cells in a monolayer. This is largely due to the anchorage dependence of mammalian cells. It appears that virtually all normal human cells must be firmly attached to some suitable surface before they will grow, multiply, and eventually secrete any products. Attempts to grow large numbers of cells in the inside surface of roller bottles⁸ and multiplate propagators⁹ has permitted moderate scale mammalian cell cultures.

Some additional success has been achieved from growth cells attached to spiral wound membranes and to the surface of hollow fiber dialysis bundles, in which nutrients can be supplied through the permeable membrane from media circulated on the opposite side of the membrane or hollow fiber.¹⁰ However, these techniques are still greatly restricted by poor surface-to-volume (culture medium) ratios. Some of the major problems associated with monolayer cultures, namely, (1) growth in only two dimensions (surface area); (2) cell proliferation limited by depletion of oxygen and nutrients; (3) difficulties with media circulation and volume-to-cell number ratios; (4) cell/product recovery; and (5) contamination control are more readily solved by using suspension culture. Unfortunately, almost all human cells of pharmaceutical interest grow quite poorly in modern suspension culture systems. Even when suspension culture of mammalian cells is accomplished on a small scale the maximum density of cells varies between 5×10^5 to 3×10^6 cells per cm^3 . This concentration is orders of magnitude less than the number achieved in microbial cultures. Since the amount of product formed under proper physiological conditions is directly proportional to the number of the cells, high cell concentrations are critical to successful commercial cultures.

In addition, the necessity to maintain the cells in a rather dilute nutrient media allows sedimentation to occur easily which in turn requires stringent mixing. Often the cells cannot tolerate the severe shear forces caused by even the best designed agitation systems. Prolific cells growth is also limited by the oxygen supply. Sparging of air is usually the best method to supply oxygen; however, the bubbles rise and coalesce so quickly that dissolution of oxygen is often incomplete during the bubbles' brief residence in the culture media. Vigorous bubbling of air causes high shear rates and too often produces uncontrolled foaming. Foaming in turn carries cells up out of the suspension where they dry out and die. Toxic waste products, cell fragments, and autolysis enzymes then fall back into the media where they dry out and die. Toxic waste products, cell fragments, and autolysis enzymes then fall back into the media where they poison the culture. Surfactants can be used to reduce foaming; however, their application is limited because they themselves are deleterious to living cells.

In an attempt to overcome the surface-to-volume disadvantages small (150-180 micron) Sephadex or DEAE cellulose beads were developed as microcarriers for cultured cells.^{11,12} Cells are allowed to attach to the surface of the bead, then the beads are suspended in the culture vessel. This technique helped solve the attachment dependence problem of human cell culture; however, the particular surface of the bead must generally be custom designed to the requirements of the specific cell to be grown. Sometimes the surface coating also acts as a repository for accumulation of cell waste products which in turn limits cell longevity.

We have compared several of the new culture technologies with the well established monolayer culture systems using several types of mammalian cells. This experience gave insight into selected features of each technique which may be combined into a suspension culture system for mammalian cells. We also have tested all of the commercial microcarrier beads (Cytodex, Superbeads, etc.) now on the market; however, sedimentation is still a problem especially after the cells are attached to the beads. Foaming problems from sparging of oxygen also remain. Often the mechanical mixing required to keep the beads suspended is so severe that cells are damaged and dislodged from the bead surface by high shear or bead-to-bead collisions.

Comparisons of commercially available microcarrier beads and discussions in cell culture workshops indicate that there may be reasons to study cell culture technology under conditions of microgravity.^{6,7} The elimination of gravity dependent sedimentation of cells and bubble buoyancy may provide new insight into careful control of culture environments. Gentle mixing to ensure adequate mass transport could be achieved while maintaining precise control of local temperature, pH, dissolved oxygen, shear, nutrient mixing, etc., in a manner heretofore not possible in Earth-based laboratories. Table 1-2 is a list of studies and workshops conducted by the Johnson Space Center to identify research topics and experiments which are needed to support the development of a suspension type space bioreactor to be used for precision controlled cell culture studies in microgravity.

RELATED SPACE EXPERIMENTS AND CELL CULTURE SYSTEMS

Studies of potential advantages of suspension cell culture technology in micro-g will only be avoided if any direct effect of this environment on cell function is well understood. Early experiments with cells growing under weightlessness indicated that the cell density of S. typhimurium was significantly higher than the one obtained in the ground control experiment.¹³ The most noteworthy of several interpretations include the random distribution of cells in the culture liquid resulting in enhanced efficiency of nutrient transfer into, and waste transport from, the cells. There also could have been some improvement in the oxygen supply due to differences in gas-liquid

mixing. Only one well controlled experiment has been carried out in growth of human WI-38 cells during the Skylab mission.¹⁴ Normal growth was observed along with normal mitotic index and subcellular structure. There was a small change in glucose utilization, however. This was considered inconclusive by the investigators.

Experiments on Spacelab 1 showed over a 95% reduction in the ability of human lymphocytes to respond to ConA mitogen.¹⁵ A change in glucose consumption was also noted, but not considered significant until experiments could be repeated with an on-board one-g control. Other experiments showed an increase in proliferation of HeLa cells, chicken embryo fibroblasts and mitogen stimulated lymphocytes of up to 30% due to hypergravity of 10-g while glucose utilization rate was unchanged from one-g.¹⁶ Experiments on Shuttle missions 7 and 8 were performed to determine the attachment efficiency of normal human kidney cells to collagen-coated microcarrier beads.

It was expected that the attachment rate would be reduced after mixing in microgravity, since the only opportunity would be random collisions while the cells and beads floated free in the culture medium. However, the results showed a significant increase in attachment for the flight samples versus the ground control experiment.¹⁷ Within 3 hours, the cell attachment (based on the average number of single cells per bead) was 50% greater in microgravity than on Earth. Once attached, both the flight and ground control cells grew at the same rate during the first 25 hours. Cell-to-cell attachment was also greater among the cells free-floating in weightlessness. Future experiments are planned to give greater insight into the attachment mechanisms. However, practical implications include the possibility of seeding microcarrier cultures in microgravity and the knowledge that cells could reattach to microcarrier if they come off the bead surface in slowly mixed space bioreactors.

CONSIDERATIONS FOR CULTURE OF MAMMALIAN CELLS IN MICROGRAVITY

The basic approach of NASA scientists to determine what factors must be included in the design of detailed cell culture experiments was to have industrial bioreactor experts study the merits of suspension cell culture systems and their applications in space. The definitive study¹⁸ was performed by Drs. Nyiri and Toth at Fermentation Design, Inc., in 1976. They recommended a perfusion reactor system with an external, cell-free, medium-circulation loop to provide resupply of oxygen, nutrients, and scavaging of waste products using hollow fiber dialysis type membrane systems. The studies specified that the development of a dedicated microprocessor control system was essential to the proper operation and data acquisition required during space flight. Further, they evaluated the commercial potential of such a system and identified several hormones and cell secretory products as valuable enough to warrant the extra costs of production in space.

Table 1-3 summarizes the general approach taken by the NASA bioprocessing program in the development of the first space bioreactor.

Based on the interest generated by these studies and the results of a 1976 Colloquium on Bioprocessing in Space,¹⁹ a formal proposal to develop a space bioreactor flight unit was approved by NASA in 1978. Thereafter, several workshops were held with researchers to define experiments and typical science requirements for the design engineers. Major conclusions of the cell culture workshops identified several areas wherein the absence of gravity dependent phenomena in the culture environment could provide unique insight into certain cell functions and interactions between the cell and its micro-environment. Table 1-4 shows the various areas where scientists believed that cell culture in micro-g could provide new information on the basic biodynamics of mammalian cells.

The objectives of the demonstration flight experiments are shown in table 1-5. Growth of normal human cells was selected because they are anchorage dependent and extremely fragile. Therefore, these cells are very difficult to grow on Earth in suspension culture systems. In space potential advantages of lack of sedimentation and bubble buoyancy could enable operations with very gentle mixing and minimum turbulence to reduce shear effects on the cells. The basic objective also included exploration of product harvesting techniques and principles of basic cell culture operation under microgravity conditions.

NASA and academic scientists then began a research program to study the specific gravity related problems which affected traditional and new cell culture techniques involving biosynthesis of cell secretory products. Research reactors were developed at two institutions and differences in gravity effects on the process technology of culturing cells which grow freely in suspension were contrasted with problems related to the anchorage dependent cells. Table 1-6 shows these steps and the basic consideration for the initial design of the space bioreactor. Table 1-7 illustrates more details of the systems development and biological systems (enzymes of cells) used to test the efficiencies of the prototype devices throughout three phases of development. As refinements were made, sensors added, and mixing techniques tested, various industrial process control systems were tested. Cultures were maintained for up to 14 days with L1210 cells; however, commercial process control systems were found to be inadequate to maintain the precise control required by human cells.

SPACE BIOREACTOR DEVELOPMENT

In recent months, the bioreactor test unit (BTU) has been designed and fabricated to be accommodated in a volume equivalent to two or three middeck

lockers on the Shuttle. Current concepts call for an early flight test of the basic unit and sensor systems to verify operations with fast enzyme reactions before a major flight experiment is conducted with live kidney calls. Designs are also considering accommodations in the Spacelab racks or special middeck experiment racks.

The basic functional requirements for culturing human cells on microcarrier beads for 7 to 10 days on-orbit are shown in table 1-8. Unique considerations include no gaseous headspace in the reaction vessel, sensors in the medium circulation loop (to eliminate turbulence in the reactor vessel) and flow/pressure controls to maintain pressures at or below 20 psia (to eliminate effects of pressure on cell functions). Practical considerations are also included based on our experience with limited access to biological experiments in the middeck while the Shuttle is on the launchpad.

Design and operational requirements for major components of the system are listed in table 1-9. The culture vessel design is based on an adaptation of two innovations by industrial researchers. We decided to use our own version of a spin filter type culture system originally designed by Thayer at Arthur D. Little Co.²⁰ This allows continuous removal of cell-free medium from the reactor vessel. We also decided to separate the mixing and spinning filter functions by using separate magnetic drives which allows independent control needed for very slow stirring during the flight portion of the experiment. The vessel volume was limited to 500 ml because of the difficulty in maintaining a large supply of human epithelial kidney cells from the same lot to reduce interexperimental variations. The target shear range for on-orbit operations was determined previously by researchers at Rice University in flow chamber studies of shear effects on kidney cells which secrete urokinase.²⁰ The cell number to medium ratios must be kept within 125% of the most effective static cultures to ensure that the cells will be able to condition the medium for maximum viability and product secretions. The mixing device chosen was based on a recent design by Feder and Tolbert at Monsanto Company²⁰ using flexible spiral vanes which helped to minimize turbulence and bead-to-impellor collision damage. Our designs call for a co-axial arrangement of the flexible vanes and the spin filter with special designs to allow high spin rates on the filter needed for high flow rates (80-100 ml/min) of medium withdrawal from the reactor vessel. This unique design is complemented by provisions for alternating backwash routes using the medium return flow to keep the spin filter from clogging.

Process control considerations include a custom microprocessor control system to control the bioreactor to set point values, automatically log sensor data, and provide minimum interface with the flight crew. Our requirements include the capability to interface with the process controller with a small personal computer (via RS-232) which in turn can operate offline to perform statistical analysis of data, and prediction of adverse trend interception of alert

or alarm set points. The capability to update the process controller with software commands from the "supervisory computer" has also been designed into the system.

The flow diagram of the reactor vessel, fluid loop, oxygenator, and the protein concentration sideloop is illustrated in figure 1-1. Detailed designs will be considered in later papers by Cross and Bowie; however, special mention should be made for position of the sensor blocks which allows calculations of cell metabolism from the difference in DO_2 and CO_2 levels in the input and the outflow from the reaction vessel. Provisions have been made to add concentrated culture medium, acid or base for pH control, dialysis type hollow fiber refeed system (which also can act as a repository for accumulating metabolic wastes). A microgravity bubble trap has also been included to remove any bubbles formed by gas dissolution during temperature or pressure change and to trap any gas bubbles which may be injected into the medium circulation loop should the oxygenator membrane develop a leak during the flight. The function of the high molecular weight filter loop is to remove serum proteins from growth medium before changeout to maintenance medium when cells are confluent and to periodically (every 3 or 4 days) concentrate a sample of the circulating medium for product assays.

PLANNED FLIGHT TESTS

The initial flight test requirements for the first two missions are shown in table 1-10. The selection of human kidney cells is based on some 8 years of in-house experience with those epithelial cells which produce urokinase, one of the few pharmaceuticals approved for production from normal human cells grown in mass culture. Also, our laboratories have extensive experience with detailed methods to screen cell lot to ensure that they are normal, healthy, and hardy enough to withstand the rigors of space flight experiments. These cells have also been flown in space several times by our research group involved in separation of kidney cells in space by free-fluid electrophoresis techniques.

Several concepts for science objectives of planned early flight tests of the space bioreactor are listed in table 1-11. The major emphasis is on culture in environmental regimes which are impossible in one-g. These include cells maintained in a very low shear field provided by slow gentle mixing which is possible in microgravity. Another tantalizing area is the culture of cells in the liquid phase of a controlled foam or bubbly culture medium comprised of oxygen bubbles which have no buoyancy nor do they coalesce readily in microgravity. Other suggested experiments involve studies of artificial oxygen carrier emulsions and specific cell physiology under quiescent culture conditions in space. More details are provided in appendix A of the Draft of the

Space Bioreactor Science Requirements Document which was handed out for reference at this meeting.

Finally, it should be obvious that experiments involving human cell cultures and suspension bioreactors will provide basic engineering data on the design considerations required for most apparatus to culture cells in the absence of gravity. Once these principles are understood, cell culture systems can be developed to provide live cells on-orbit for cell biology and other types of bioprocessing related studies in microgravity. Should the expected advantages of micro-g be great enough, it is possible that a space bioreactor could become the feeder facility in a multistep bioprocessing system, wherein the cell products would be harvested from the bioreactor and fed to a continuous flow electrophoresis system or other product purification device which can isolate pure pharmaceuticals direct from concentrated culture medium. Already the second phase of this type of bioprocess in space has been demonstrated by commercial CFES purifications on STS-4, 6, 7, 8, and other flights. Such a multistep bioprocess is a good candidate for commercially orientated research on the U.S. Space Station.

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TABLE 1-1.- CELL CULTURE RELATED RESEARCH AREAS (DATA IS NEEDED BOTH TO IMPROVE CULTURE TECHNOLOGY ON EARTH)

Microgravity Related Research Needed:

Upper limits of shear stress on cells
 Minimum shear required for adequate mixing and mass transport
 Details of required microenvironment surrounding the cell
 Effects of shear on gas transport and cell membranes
 Alterations (if any) of transport when bubbles don't coalesce
 Mass transport in the absence of thermal convective mixing
 Causes for attachment dependence and cell energy diverted to this behavior

TABLE 1-2.- BIOREACTOR STUDIES

1976 - 1977	"Space Biosynthesis Systems" - Contact NAS9-14961 by Fermentation Design Inc., Bethlehem, Pennsylvania. Investigator: L. K. Nyiri, Ph.D. and G. M. Toth, Ph.D.
1977	Proposal to Develop Experiments for Multipurpose Space Bioreactor System. Proposal #770056, NASA A.O. No. AO-77-3, L. K. Nyiri, Ph.D. and Marvin Charles, Ph.D.
1978 - 1979	"Development of a Space Bioprocessing System for Cell Culture." Contract NAS9-15619, Lehigh University, Bethlehem, Pennsylvania. Marvin Charles, Ph.D. and L. K. Nyiri, Ph.D.
1979	"Cell Culture Biosynthesis." Contract NAS9-15322, A. D. Little Co., Cambridge, Massachusetts. Phil Thayer, Ph.D. and M. G. Broone, Ph.D.
January 1978	M.I.T. Workshop on "The Feasibility of Cell Culture Systems for Space Experiments." Proceedings summarized by A. D. Little Co. Inc., - Final report NAS9-15322.
June 1979	Salk Institute Workshop on "Microgravity Cell Culture Systems." Proceedings summarized by A. D. Little Co. Inc., - Final report NAS9-15322.

TABLE 1-2.- Continued

January 1982	JSC Conference on "Fluid Mechanical Aspects of Cell Culture," Rice University. Summary report by Mike Reynolds, Ph.D.
1982	"A Microprocessor Interface for the Space Bioreactor System." Conducted by Robert A. Synder, Ph.D., (NRC Postdoc).
August 1985	JSC Workshop on "Space Bioreactor Science." A joint NASA, academic, and industrial workshop on the development of a space bioreactor and potential flight experiments. Proceedings published in JSC document no. 22264, edited by D. R. Morrison, Ph.D., August 1986.

TABLE 1-3.- SPACE BIOREACTOR

Approach

- Conceptual studies by academic institutions and industry started in 1976
- Cell culture sciences conferences - MIT, Salk Institute, JSC
- Industrial consultation with: Monsanto Corp.; Stanford Research Institute; MDAC-St. Louis; Biochem Technologies; Fermentation Design, Inc.; K. C. Biologicals; Bioreactor, Inc.; Tissue Culture Association; Alton Jones Cell Science Center; and Arthur D. Little Co.
- Academic meetings with: MIT, Princeton University, University of Rochester, University of Mississippi, Stanford University, Rice University, University of Houston, and Washington School of Medicine
- JSC has accumulated 8 years experience with cell cultures and 4 years experience with bioreactor breadboard system
- JSC will develop two space bioreactor laboratory test units which will confirm design requirements for flight units and can be used for hardware verification flight tests (DSO's)

TABLE 1-4.- BIODYNAMICS

Micro-g environment research on

- Shear stress on cells
- Anchorage dependence - cell attachment
- Gas transfer/gas-liquid foams
- Nutrient/waste diffusion
- Intercellular transport
- Growth
- Bioenergetics
- Product secretions

TABLE 1-5.- SPACE BIOREACTOR

Objectives

- Develop and test prototype system for controlled growth of mammalian cells in microgravity
- Demonstrate advantages
 - Lack of sedimentation
 - Lack of bubble buoyancy
 - Unconventional controlled mixing at slow speeds to keep shear at minimum
 - Culture of extremely fragile cells
 - Precise control of gas/liquid transport, nutrients, and waste
 - Evaluate new methods of O₂ control
- Develop continuous culture methods and product harvesting techniques
- Provide a basic cell culture and maintenance facility for support of cell biology experiments (Shuttle, Spacelab, and Space Station)
- Provide a testbed capability to explore other types of cell culture including hybridoma cultures, yeast fermentation, etc.

TABLE 1-6.- BIOSYNTHESIS

Gravity Problems Identified

- Comparison of culture techniques
 - Monolayer/multiplate
 - Hollow fiber
 - Spin filter suspension
 - Microcarriers

Development of research bioreactor

- Suspension cultures
 - L1210
 - Hybridoma
- Attached human cells
 - Kidney

Development of space bioreactor

- Spin filter/microcarriers
- No headspace
- Micro-g fluid handling

TABLE 1-7.- SPACE BIOREACTOR

Phase I - Breadboard

<u>Systems Development</u>	<u>Biological Test System</u>
Spin filter system - (no headspace)	Enzyme reactions
Sensor qualification	● Nutrient consumption
Fluid circulation loop	● Nutrient supply
Temperatures control	● Culture medium components
O ₂ , CO ₂ - gas input systems	
Sterilization	

Phase II - Research Bioreactor

<u>Systems Development</u>	<u>Biological Test System</u>
Refined sensor development data logging	Suspension culture - L1210 cells
Nutrient consumption	● Maintenance requirements less
Growth and upper limits to cell mass	● Less complex culture media
Stir/mixing techniques	● Easier for cell counting
Microprocessor feedback control	Attached cells - microcarrier beads

TABLE 1-7.- Continued

Phase III - Research Bioreactor

<u>Systems Development</u>	<u>Biological Test System</u>
<p>Bioreactor test unit (middeck)</p> <ul style="list-style-type: none"> ● Culture chamber + fluid circ. loop ● Minimum sensors recorded data on-orbit <p>Bioreactor - flight unit</p> <ul style="list-style-type: none"> ● All up system/microprocessor control ● Precision control for human cells attached to microcarrier beads ● 1 Spacelab rack or 3 middeck lockers 	<p>Enzyme reaction</p> <ul style="list-style-type: none"> ● Fast reaction to test sensor and set limit type feedback control <p>Suspended cells (L1210)</p> <p>Attached cells</p> <ul style="list-style-type: none"> ● Human kidney cells on beads ● Pituitary cells on beads

TABLE 1-8.- BIOREACTOR: BASIC FUNCTIONAL REQUIREMENTS

System Requirements

- Basic design - cells on microcarriers
- No headspace in reactor vessel
- Cell/bead free media circulation loop
 - Oxygenation
 - Resupply nutrients
 - Remove toxic waste
 - Product removal
 - In-line sensors
- Autoclave entire core bioreactor
- Pressure on cells <-- 5 psi
- System pressure - up to 15 psi
- Core bioreactor loaded at KSC/installed at L-10 hours
- Self-sufficient process controller - standard operations and alarms
- Separate supervisory computer
- Target core bioreactor - 2 lockers
- Process controller/support electronics - 1-2 lockers

TABLE 1-9.- MAJOR COMPONENT REQUIREMENTS

<u>Reactor vessel</u>	<ul style="list-style-type: none"> - Separate devices for spin filter and mixing - 500 ml culture volume - Minimum internal components - Control maximum shear 10-40 dynes/cm²
Approach	<ul style="list-style-type: none"> - Basic test vessel - spin filter (must be kept clean) - Marine impellor vs. spiral vanes
<u>Process controller</u>	<ul style="list-style-type: none"> - Onboard CPU/operating system - Log all data 12-24 hours - Minimum trend projections - Interface (RS-232) with supervisory computer - Minimum crew interactions - Update with software commands
Approach	<ul style="list-style-type: none"> - Test commercial systems - none adequate - Build custom system - Buy MDAC ECCM
<u>Supervisory computer</u>	<ul style="list-style-type: none"> - Off-line data analysis - Complicated trend projections - Graphics
Approach	<ul style="list-style-type: none"> - IBM-AT (ground) and lap computer for flight

TABLE 1-10.- BIOREACTOR TEST REQUIREMENTS

<u>First flight test</u>	<ul style="list-style-type: none"> - No cells - Minimum contamination control - Test - fluid system <ul style="list-style-type: none"> - Mixing - Sensors - HMW filter system
<u>Second flight test</u>	<ul style="list-style-type: none"> - Culture feasibility demonstration - 7-8 day mission - Kidney cells on microcarriers - Grow on beads before flight (growth medium) - Maintain cells on UKPM (serum free) - Exercise HMW filter on 4-day intervals - Replenish medium with new medium or dialysis refeed

TABLE 1-10.- Continued

- Later flights
- Grow cells on microcarriers, then switch to serum-free medium
 - Other reaction vessels
 - Hybridomas
 - Fermentation (yeast)

TABLE 1-11.- EXPERIMENTS WITH HUMAN CELLS (ATTACHED TO MICROCARRIERS)

Effects of shear stress

- Low shear 10-40 dynes/cm²
- Target exact shear range to stimulate UK secretions
- Direct measure of metabolism - fluromasure system

Culture in a controlled foam in the absence of buoyancy

- Create stable foam of O₂ bubbles in culture medium
- Grow cells in liquid phase
- Provide continual and intermediate access to O₂ supply without cells ever being exposed to gas phase
- Completely avoid use of anti-foaming agents

Use of fluorocarbon oxygen carriers in absence of density-driven separation

- Use of perfluorinated carbon solvents (S.G. 1.87)
- Maintain a long-term stable dispersion to carry O₂ from oxygenator to the cells in reactor vessel
- Explore use of serum in media where foaming will not be present as in Earth-based systems

OTHER EXPERIMENTS TO DETERMINE DIRECT CELLULAR EFFECTS

- Alterations of cell physiology or morphology
- Cell secretions in microgravity - GH, UK
- Cellular effects of magnetic fields in absence of gravity
- Alterations in cytoskeleton, cell motility

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OF POOR QUALITY

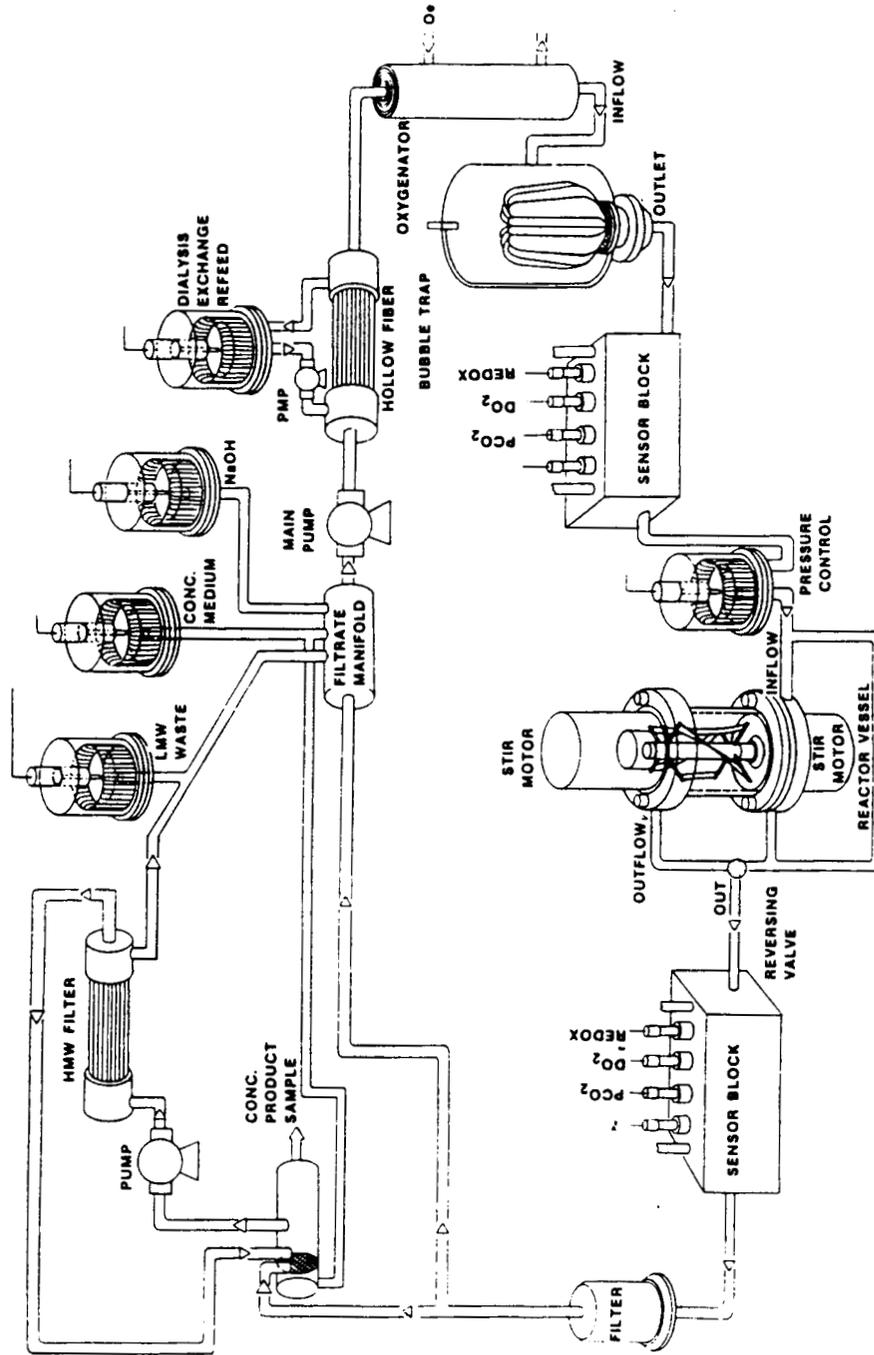


Figure 1-1.- Space bioreactor main components and fluid loops.

SPACE BIOREACTOR: DESIGN/PROCESS FLOW

John H. Cross
Technology Incorporated

The design of the space bioreactor stems from three considerations. First and foremost, it must sustain cells in microgravity. Closely related is the ability to take advantage of the weightlessness of microgravity. Lastly, it should fit into a bioprocess such as shown in figure 2-1. This paper will describe the design of the space bioreactor in view of these considerations. The flow chart of the bioreactor will then be presented and discussed.

The conceptual space bioprocess in figure 2-1 yields pure product after six steps. The initial step is to select the cells that produce the most product. Studies already conducted in space have shown that some cell fractions selected by electrophoresis produce more of a given substance than others. The next step is to prepare a bioreactor inoculum from these cells.¹ This step is shown in a dotted box, because it has not been investigated in space. The third and fourth steps are those covered by the space bioreactor. The bioreactor design consists of two loops. The first loop contains the cell culture vessel and all the apparatus necessary to sustain the cells. The second loop, isolated from the first, has an ultrafilter or similar device for separation of the product from the cell culture media. The fifth step, again not tested in space, is concentration to the point the solution of product can be introduced into a continuous flow electrophoresis system for final purification. This last step has been quite well developed by the McDonnell Douglas Astronautics Company, Inc.

An important driving force for building a space bioreactor is clear from figure 2-1. The first and last steps are more efficient in space. Connecting the two with a space bioreactor makes possible a fuller exploration of the advantages of space for manufacturing pharmaceuticals. It should also be pointed out that the cells for the first step could be grown in space in the bioreactor.

The next consideration in the design is to take advantage of microgravity. Cells cultured in the weightlessness of space will not sediment. The only stirring required is to distribute oxygen and nutrients to the cells. This amount of stirring is expected to be considerably less than is needed to keep the cells suspended in 1-g. Less stirring means less turbulence and a lower rate of cell death. Consequently, it is hypothesized that in space higher cell concentrations can be cultured.¹ To allow for the best experimentation on this hypothesis, the cell culture vessel has few internal parts. The sensors and other parts are instead placed in the first loop mentioned above. The medium is pumped around the loop and passes over sensors for oxygen,

carbon dioxide, and pH. The oxygenator and other necessary vessels are also in this loop, called the main medium circulation loop. The reactor vessel has in it only a stirring vane and a spin filter, which keeps the cells in the reactor.

What type of cells would benefit most from a minimal stirring situation? After an assessment of different cell culture systems, it was concluded that the answer was mammalian cells on microcarrier beads. Specifically, human embryonic kidney cells have been selected for the first flights because they have been separated in space by electrophoresis. These cells produce urokinase, a pharmaceutical that dissolves blood clots. Many other types of mammalian cells could be cultured with minor procedural modifications. Details of the reactor design reflect the culture requirements of mammalian cells.

A second advantage of microgravity is expected to come from the stability of foams in space.² On Earth a foam of a gas in a liquid separates rapidly as the gas rises to the top. In space this density-driven separation does not occur and surface tension forces the gas bubbles from coalescing. One proposed experiment is to study oxygenation of a cell culture with a foam of tiny air or oxygen bubbles. The modular bioreactor design, a circulation loop connecting specialized vessels, allows for insertion of the apparatus needed for this experiment. Hopefully, this flexibility will prove to be general. Other experiments might be proposed after the data from several flights of the bioreactor clarify the effects of microgravity on cell culture.

The combination of operating in microgravity and in the Shuttle Orbiter vehicle places certain constraints on the bioreactor design (table 2-1).

Headspace will not exist in the cell culture vessel in microgravity. Many bioreactors use this headspace as a reservoir for oxygen and carbon dioxide. In the space bioreactor, the vessel is kept full of medium. Without headspace, the bearings for the spin filter and the stirring vane are immersed in the medium. Consequently, a potential exists for grinding cells between the bearing surfaces. Designs for the cell culture vessel include versions with sleeves that cover the bearings and versions that route cell-free medium through the bearings.

The remainder of the items in table 2-1 are operational constraints. Complete liquid containment is mandatory, because spills in space cannot be easily cleaned up; the bioreactor uses magnetic drives to minimize points for leaks and stainless steel tubing instead of plastic tubing. To minimize the possibility of contamination during operation in a nonsterile environment, the bioreactor is autoclaved as a sealed unit. It is opened only to charge it with medium and cells. The bioreactor must be compact and energy efficient to allow the maximum number of experiments to be carried out on each

Shuttle flight. The lessons learned from these design constraints may give industrial biochemists clues for reducing capital and operating costs. Automatic operation of the bioreactor is another consequence of trying to maximize the number of experiments on every flight; the crewmembers can spend only so much time with every experiment. The space bioreactor design includes an advanced process control scheme (described later) that provides for operating the system, logging data, and controlling the dynamics of a biological system.

This completes the discussion of the design considerations for the bioreactor. The next subject is a description of the flow chart. To begin this, table 2-2 summarizes the essential elements of any bioreactor. The following discussion will show how these elements are expressed in this particular design. Detailed descriptions of individual components are given in the paper by William Bowie.

The culture vessel, labeled reactor vessel, is a 500 ml perfused vessel with a new version of a spin filter from Virtis Company (fig. 2-2 and table 2-3). The cells are, as stated, anchored on microcarrier beads and suspended in the culture vessel. To get the desired gentle mixing, a stirring vane similar to that described by Feder and Tolbert³ will be tested initially. The reactor is designed to operate with no headspace. Medium from the main medium circulation loop, Stream 1, is pumped into the reactor vessel and withdrawn through the spin filter, which allows the medium to pass, but retains the microcarrier beads with the cells. Locating sensors for pH, dissolved oxygen (dO_2), CO_2 , and redox potential before and after the reactor in the medium circulation loop provides for differential measurements across the reactor and reduces turbulence and shear within the reactor.

In Stream 1 are vessels for oxygenation, separation of depleted medium (upper left, Waste or Sample Tap), replenishment of medium, and pH control. A connection to the product separation loop, Stream 3, is also provided. A dialysis unit to separate wastes is contemplated, but has not been incorporated. Provision is made to maintain the entire loop at $37^\circ \pm 1^\circ C$. The oxygenator has a membrane that is permeable to oxygen and carbon dioxide. A gaseous mixture of nitrogen and oxygen (possibly room air) is passed through one side of the membrane. Carbon dioxide from metabolism passes from the medium to the gas mixture and is carried away. This design allows for addition of CO_2 if desired. System pressure is designed for 5 psig with a maximum of 15 psig; four transducers are incorporated in the system. Concentration of the medium is done in Stream 3 by ultrafiltration or hollow fiber filtration. This loop will be run every 3 or 4 days to provide a solution of urokinase and other cell products for analysis. (In a complete bioprocess, this loop would be designed to provide crude urokinase to a purification unit, figure 2-1, steps 5 and 6.)

The process control scheme has two levels of control (fig. 2-3). The level depicted at the top maintains set levels of pH, dO_2 , CO_2 , and temperature. These variables determine the environment of the cells. The environmental controller is being built in the JSC labs from a STD bus system. This system has been used successfully for the McDonnell Douglas continuous flow electrophoresis system and the 3M crystallization unit. In addition to operating the system, the STD bus system logs data from the biochemical sensors, the flow meters, and the pressure transducers. This part of the process control system will fly. In many processes, controlling the environmental variables would control the process. A biological system, however, is more complex. To obtain the extra control required, a second level of control has been designed to monitor the metabolism of the cells.

This function, termed an "expert system," will supervise the process controller by adjusting the environmental set points. The metabolic parameters now thought to be the most important are the respiratory quotient (dO_2/CO_2 concentration ratio), concentration of NADH, and the type and quantity of cell secretory products, such as urokinase. Some of the data used by the expert system will be from online sensors, but results from reactor samples and subjective evaluations of previous experience will be incorporated using "fuzzy modelling."⁴ An IBM AT computer will be used. The expert system computer will be used for development and other ground-based operations. More details are given in the paper by Bill Hall.

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TABLE 2-1.- HARDWARE CONSTRAINTS

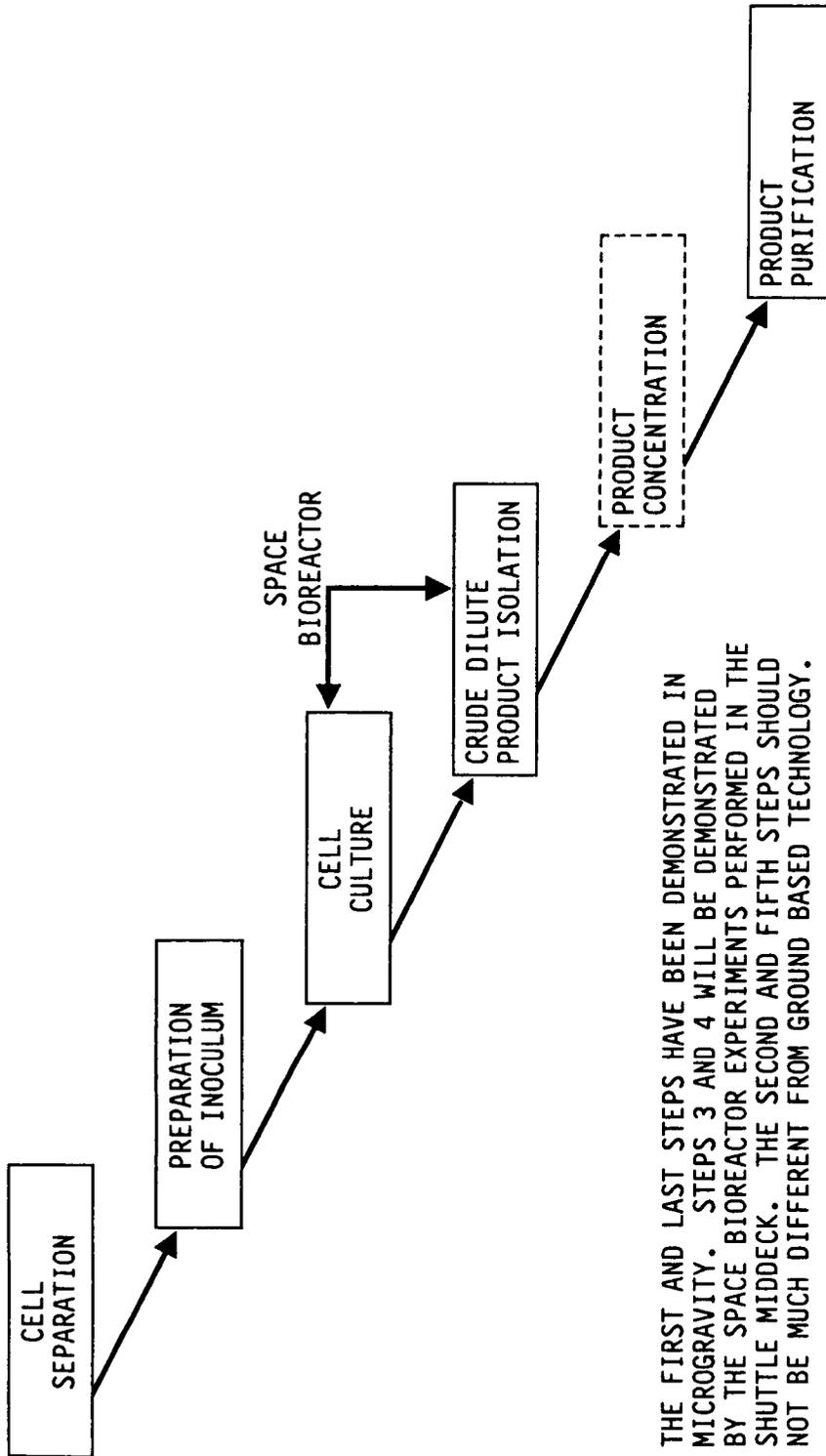
No Headspace
Complete Liquid Containment
Autoclave
Compact
Energy Efficient
Automatic

TABLE 2-2.- ESSENTIAL ELEMENTS

Oxygenate
Resupply Nutrients
Remove Wastes
Remove Product
Collect Biochemical Data

TABLE 2-3.- STREAM DESCRIPTIONS FOR FIGURE 2-2

<u>Stream No.</u>	<u>Description</u>	<u>Composition</u>	<u>Flow Rate</u>	<u>Remarks</u>
1	Main medium circulation loop	Cell free medium	50-100 ml/min	This stream provides all the cell maintenance requirements. Composition adjusted by process controller.
2	Feed to product separation loop	Cell free medium	As needed	
2a	Flow restriction device	Below device, same as 1. Above, same as 3.	-	Device provides for adding Stream 1 to Stream 3.
3	Product separation loop	Medium, relatively high product concentration	180-200 ml/min	Operated every few days to concentrate medium sample, which is withdrawn for in-flight product assay.
4	Return stream from product separation loop	Medium, depleted of product	Low, 20-30 ml/min	Can be diverted to waste vessel.
5	Oxygenator feed	5-10% O ₂ 0-5% CO ₂ N ₂ balance	Flow essentially unrestricted	-
6	Oxygenator effluent	O ₂ (excess) CO ₂ from metabolism N ₂	Flow rates same as 5	-



THE FIRST AND LAST STEPS HAVE BEEN DEMONSTRATED IN MICROGRAVITY. STEPS 3 AND 4 WILL BE DEMONSTRATED BY THE SPACE BIOREACTOR EXPERIMENTS PERFORMED IN THE SHUTTLE MIDDECK. THE SECOND AND FIFTH STEPS SHOULD NOT BE MUCH DIFFERENT FROM GROUND BASED TECHNOLOGY.

Figure 2-1.- Conceptual bioprocess.

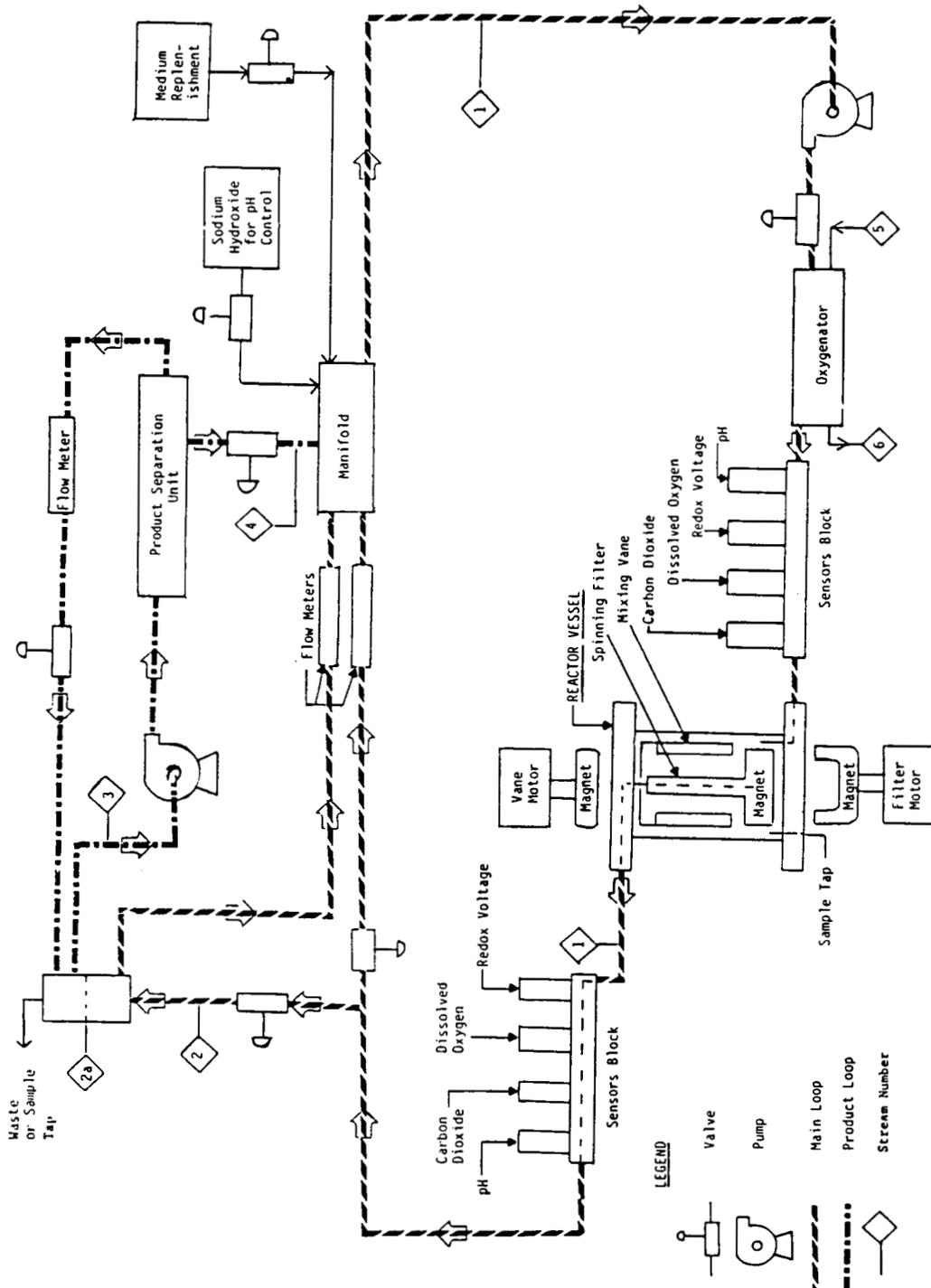


Figure 2-2.- Space Bioreactor flow chart.

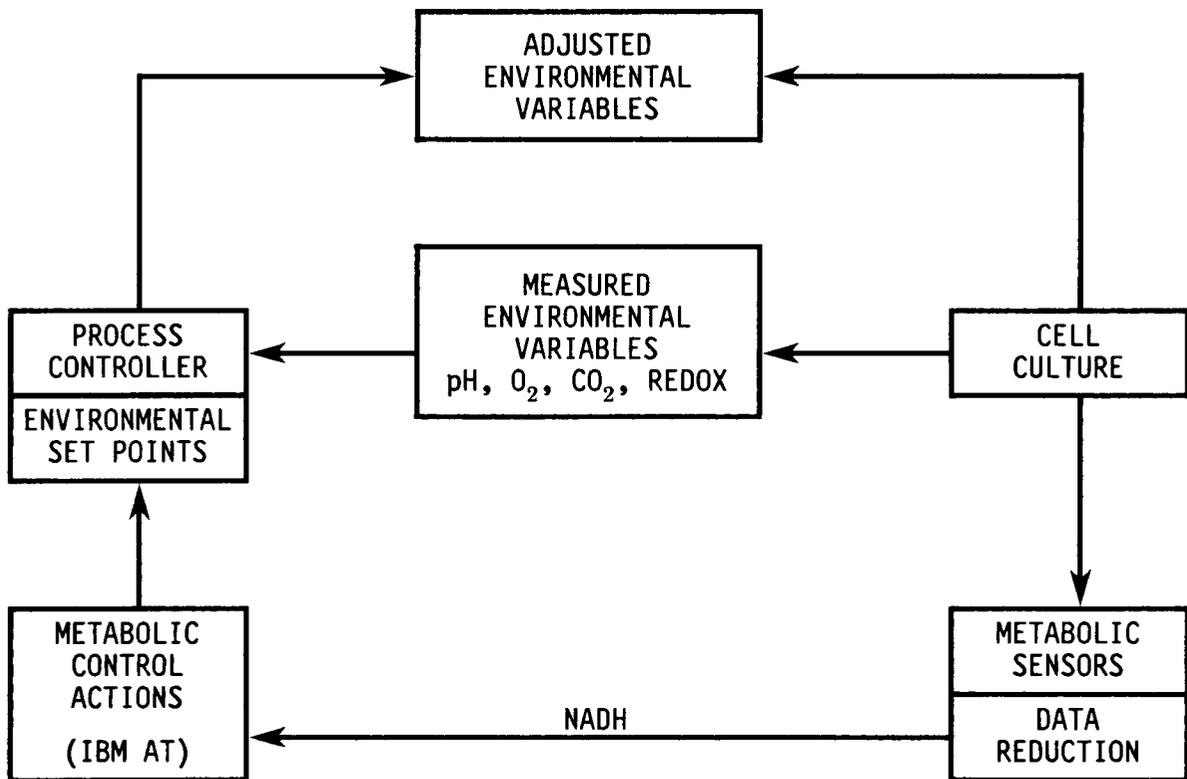


Figure 2-3.- Process control scheme.

BIOREACTOR DESIGN CONCEPTS

William Bowie
Technology Incorporated

INTRODUCTION

Serious development of a space bioreactor was begun at JSC in the spring of 1982 under direction of the Principal Investigator (D. R. Morrison, JSC). The work was an outgrowth of earlier studies by Martin Marietta, Arthur D. Little, and Fermentation Design. The results identified the concepts for suspension cell culture in a zero-g environment.

The first two years of the program were devoted to development and proving of concepts. Two of those concepts which received the most work were "zero headspace" and diffusion oxygenation. Other areas of research were sensor technology, pump operation, sterility, and culture maintenance. Work during those first years was conducted with mouse lymphocytes, strain L1210. Those cells were chosen because of their ease in culturing, then low cost and availability. We did not think it prudent to utilize an expensive, hard-to-obtain cell line during early development work.

The reasons and rationale for the construction of a space bioreactor are not covered in this paper. Those topics are detailed in the first paper (Morrison) presented at this bioreactor workshop. This paper presents some of the developmental logic and considerations which have led to the present successfully operated laboratory version of the space bioreactor.

BIOREACTOR-EVOLUTION

Bioreactor systems techniques and equipment have undergone significant changes since initiation of the program. Due to the developmental nature of the reactor system, such changes can be expected and are a normal part of the system evolution.

Bioreactor equipment and technique changes have been incorporated in the following systems:

- Pressure control system
- Fluid flow
- Gas input control
- Sterilization
- Stir (agitation) system
- Oxygenation

- Culture density control
- pH control

The following are brief explanations of each system area.

- Pressure control system - Early efforts at pressure control relied on pressure gages and subsequent adjustment of pumping rates or fluid bypass flows. However, pressure gages have proved to be unsatisfactory because of their inaccessibility via electronic systems. As long as the system was sterilized by heat, cavities in such devices presented no serious problems. If heat sterilization is not desirable, contaminating organisms trapped in the cavities cannot be killed or removed without the use of strong chemical sterilants. Sterilants such as ethylene oxide or glutaraldehyde which outgas enough to penetrate empty cavities. However, absorption of the sterilant by water trapped in the cavities leads to leaching of chemical contaminants into the process system. Consequently, a substantial effort was initiated to locate pressure transducers with no dead end cavities. One such device was located and installed, but has failed to operate satisfactorily.
- Fluid flow system - Rotometers of fixed cross-sectional areas were utilized originally for flow measurements. Proper functioning of such devices was dependent upon their orientation in a gravity field. Also they were not inherently accurate devices and resulted in more fluid path length than was desirable, thus increasing the system volume.

Electronic flow transducers were ordered for the second stage reactor. Two different types of transducers were desired; one was dependent upon differential pressure across an orifice, while the other was a vane type turbine meter. The differential pressure device is used in the low flow loops where rates of 10 ml/min or less are encountered. Turbine meters are used in the higher flow loops where flow rates of 10's-100's ml/min are encountered. Both flow devices function in a zero-g environment.

- Gas input control - Our original bioreactor system relied on gas flow rates set by metering valves on the input side of rotometers. As the system pH decreased due to cell metabolism, adjustments in the CO₂ and air input rates were recognized as necessary. A common observation was a shift to alkalinity when attempting to input relatively high (>20%) concentrations of oxygen. Such shifts could be corrected by increasing the CO₂ concentration. However, the system was constructed to accommodate only two gases. Thus, if greater than 20% oxygen were required it would have to be mixed prior to entering the control valve and meter. Such a gas mixing system was not available during the early phases of reactor construction.

A more advanced reactor incorporated a gas mixing system which controlled a component gas at a fixed level relative to a carrier gas. For instance, if 5% CO₂ in air were desired, the system would deliver 5% CO₂ relative to the air flow rate. Increasing the oxygen concentration over 20% was still a problem, but one that was partially solved. A one-way check valve was installed in the air input line upstream from the air controller. Oxygen was brought into the system between the check valve and the controller at a higher pressure than the incoming air. As the oxygen tension in the reactor vessel fell below some set point (i.e., 20%), a solenoid valve opened allowing a short surge of oxygen to enter the air line. The higher oxygen pressure closed the check valve, but also increased the flow rate and pressure sensed by the controller. Consequently, control measures were automatically instituted to maintain the previously set flow rate. Some transient bounce was observed in the resulting flow, but it did not affect the gas absorption dynamics of the reactor media.

A more desirable method of gas control would have been an additional control unit dedicated to oxygen. Thus, three controllers would provide CO₂, air or nitrogen, and oxygen gas as required. Their total flow rates would be compared to the output of a flowmeter upstream from the reactor and the flow of each gas would be adjusted to maintain a desired oxygen-CO₂-air or nitrogen level.

- d. Sterilization - Considerable difficulty was encountered in the development of system sterilization techniques. Several components (i.e., probes and oxygenator) were heat labile and suffered severe performance decrements after steam autoclaving. Nevertheless, the first prototype system was sterilized via a combination of autoclaving and ethylene oxide. The system was dismantled and physically moved in pieces to the various facilities. Such procedures proved to be awkward and time consuming, but were successful.

Several chemical sterilants or disinfectants were tried and of those, a 2% glutaraldehyde proved to be successful. Thus, it was not necessary to dismantle the system except to wash it and even that could be successfully accomplished by the use of an appropriate detergent circulating through the reactor plumbing. However, a major disadvantage to the in-place washing and sterilization is the long period of post sterilization washes with sterile water to remove sterilant residuals.

- e. Stir (agitation) system - A Corning laboratory stirrer was used as the first reactor stirrer. The device had a small rotor that was prone to decoupling from the vessel stirrer. It also was difficult or impossible to regulate slow stirrer speeds (in the area of 30 rpm or lower) due to the motor characteristics.

As a consequence, a commercially available motor and controller were purchased. The motor was equipped with a feedback generator which provided shaft rpm data to the controller, thus accurately regulating stirrer speed at some dialed-in value. A large, heavy magnet was affixed to the motor shaft, thus lessening the possibility of stirrer decoupling. Speed control was very precise from very low rpm (<10) to very high rpm (>500).

- f. Oxygenation - A 4.5 m² membrane external oxygenator was first utilized; however, the device proved too large and resulted in very low through media flows. Such low flows through the oxygenator in turn caused pockets of media stagnation. A 0.4 m² membrane device was subsequently purchased and produced better results than larger units. Flow rates were higher through the device, which resulted in little or no media stagnation.

Low L1210 cell densities (to about 200,000 per ml) could be adequately oxygenated with air at 100 ml/min and liquid flow rates of about 0.5 ml/min. Higher density cell population (to about 500,000 per ml) required 100% O₂ at the above flow parameters while cell densities over about 500,000 cells/ml required an increased fluid flow.

A more efficient oxygenator system was required because of pump rate limiters through the spin filter. Consequently, a silicon membrane envelope was applied to the inside of the reactor vessel. Thus, no media pumping was required to contact the membrane. Culture densities of 1.8×10⁶ cells/ml were achieved with air plus 5% CO₂.

- g. Culture density control - Cell culture densities depend on the supply of nutrients and respiratory gases. Oxygen supply control has been discussed previously and will not be presented in this section.

Control of cell densities has been accomplished in other systems (i.e., algal and bacterial) via light attenuation or scattering techniques. As the culture increased in density more attenuation or scattering would occur and at a predetermined value, a pump would be activated to remove old culture and infuse fresh media. Such a system can be made functional on the bioreactor provided suspension cells are grown.

Use of pH indicators such as phenol red in the media complicates the light sensing circuit by introducing a color change as the culture matures. Consequently, elimination of the indicator dye is recommended for bioreactor studies. With no color change, a simple turbidostat will control suspension cell densities.

- h. pH control - Cell culture media (Fischers media for leukemic cells of mice) has a pH of about 7.2 (6.9-7.3). However, during cell growth, that pH will normally shift toward acid. An opposite shift toward alkalinity occurs in the presence of high dissolved oxygen or at room atmosphere equilibration.

Neither pH extreme can be tolerated long by the cells. So some means of controlling the hydrogen ion concentration was necessary. Such control was complicated by the 5% CO₂ routinely used in the input gas. As the culture increased in density, more oxygen and less CO₂ became necessary. Thus, the ratio of oxygen to CO₂ was changed throughout the life of the culture and that change resulted in undesirable shifts in the culture pH. In order to counteract gas and growth-induced pH changes, an external pH control loop was devised.

The pH control loop received its input from a sealed combination pH electrode immersed in the growth media. That signal was amplified by Orion pH meters and fed into a double comparator circuit. As the pH shifted toward acid or base, the voltage sensed by the culture electrode was continuously compared to a reference voltage. When the sensed voltage drifted higher than the reference voltage, a pump was activated through a solid state relay and acid (.25N HCL) was slowly added to the culture. Conversely, when the pH fell below its reference value, .25N NaOH was pumped into the culture.

THE CURRENT SYSTEM

The bioreactor consists of two major fluid loops. A third loop may be envisioned by connecting the two major loops. All three loops are shown by heavy, dark lines in figure 3-1. The primary loop routes fluid through the reactor vessel (RV) and its support equipment, while the secondary loop routes fluid through the protein extraction unit. A connecting fluid line between the extraction unit and the filtrate vessel completes the fluid circuit.

A number of different fluid pumps were considered for the bioreactor. Any pump that produced detectable pulses in the fluid flow was eliminated in order to achieve tighter control through the feedback circuits. An additional elimination factor was the presence of dynamic seals; such seals have the potential of leaking and cannot be tolerated in a sterile pressurized system. The pumps chosen and shown as P1 and P2 in figure 3-1 were positive displacement, essentially pulseless units with static seals. However, some question has been raised as to the effect of the gears on long chain, complex molecules. Studies into that area are planned, but are not yet underway.

Shown in figure 3-1 on the output side of P1 is a feature called a "high dialysis refeed" and the notation TBD. That feature represents one potential location for a media conditioning unit to be composed of some type of dialysis equipment.

Commercial fermentors have, in the past, utilized headspace and sparging for media oxygenation. Headspace cannot be tolerated in zero-g, but some sparging with microbubbles and controlled foams may be usable concepts. Our efforts to date, have been directed toward diffusion oxygenation. One such diffusion device involves the placement of a thin silicone membrane bag, envelope, or coil inside the reaction vessel, thus, diffusing oxygen directly into the cell support media. That method works well for small vessels (thus far up to about 1 liter) and eliminates the need for fast fluid flow; it also simplifies the system pumping. A second method utilizes oxygenators external to the reactor vessel.

An extracorporeal blood oxygenator is downstream from the proposed dialysis unit in figure 3-1. The oxygenator functions to provide cells in the reactor vessel with a vital oxygen supply.

The oxygenator chosen for our bioreactor is manufactured in several different sizes and is a diffusion device. Incidentally, we discovered that the same unit is used by McDonnell Douglas to degas and remove bubbles from their continuous flow electrophoresis system. However, the commercial version does not tolerate repeated use and cannot be autoclaved, thus, some modifications were necessary to adapt it to the bioreactor. Those modifications consisted of repackaging the membrane in a rigid, temperature-resistant sleeve and rerouting the gas input and output lines.

Gases are provided to the oxygenator via a set of three mass flow controllers; one each for nitrogen, oxygen, and carbon dioxide. The total gas flow is set and controlled via a mass flowmeter on the oxygenator input. Oxygen, nitrogen, and carbon dioxide ratios are adjusted via computer commands to provide an optimal environment for the cells.

The block labeled SB1, downstream from the oxygenator is a device called a "sensor block." It consists of a delrin block with a through-channel and at least four electrochemical sensors. The channel allows media to pass through the block. Each electrode is positioned such that its sensing element is in the media channel. The block contains one each oxygen sensor, carbon dioxide sensor, pH electrode, and reduction-oxidation electrode.

Several oxygen, carbon dioxide, and pH/Redox electrodes have been investigated. The oxygen electrode installed in the bioreactor was chosen by a combination of default and recognition of the state of fermentation technology. All other electrodes tested or investigated failed in some respect

to satisfy our requirements. Also, the consensus among fermentation users was that the electrode chosen was perhaps the best available. The carbon dioxide electrode was chosen because no other suitable electrode was available. Redox and pH electrodes were available from several manufacturers, but only Phoenix electrodes worked with us to develop the type of electrode required for our bioreactor system.

The heart of the bioreactor consists of the RV. That vessel contains 500 ml of cell bead suspension and the media extraction filter. The principle of tangential filtration is employed to remove media from the RV. Specifically, a spinning filter is used which, due to the sweeping motion of the fluid across the filter, results in much less filter clogging than would be encountered in a stationary filter.

A spinning filter alone will not suspend the cell bead mass, so small propellers or vanes have been used for that purpose.

A second sensor block (SB2) identical to SB1 is located on the output side of the RV.

Cell debris conceivably could find its way through the spin filter body or seals and thus be forced into the reactor fluid stream. Consequently, a stationary filter has been installed before the mixer vessel to trap particulate material before it enters the protein extraction unit.

A second primary loop vessel is a mixer vessel and serves to provide a buffer and reservoir between the relatively low flow RV system and the higher flow of the high molecular weight (HMW) extraction system.

Extraction of high molecular weight products is accomplished in the secondary loop which derives its feedstock from the mixer vessel. Pump P2 is similar to pump P1 and provides the required flow through the protein extraction unit. An optimal pressure is required for the extraction unit to successfully extract product; that pressure is provided by a bellows valve downstream from the extractor. During operation, 80 to 90% of the media passing through the extraction unit is shunted back into the mixer while the remaining few percent is bled off as waste or shunted into the filtrate vessel.

Between the extractor and the filtrate vessel is a second potential site for a dialysis unit. Both dialysis units are undefined and will remain so until analytical data detailing media depletion and waste product buildup is available.

The filtrate vessel is functionally a manifold into which fresh media is input and from which pump P1 draws media for the main loops.

A number of support vessels are necessary. Those vessels are the media reservoir, sodium hydroxide waste vessel, and pressure relief-air vessel waste vessel. No acid reservoir is utilized because during the life cycle of a cell culture, a strong trend toward acidity is normal.

The media storage, NaOH, and waste vessels employ a rolling diaphragm to provide compliance. Diaphragm position and vessel volume is determined via linear variable differential transformers attached to the piston. All vessels have the feature of being detachable from the system via quick disconnects. However, the media reservoir and NaOH vessel reside in the sterilized bioreactor system, while the waste vessel is a component of the service module and remains outside the system.

Pressure relief and air waste is provided by a small vessel, about 250-300 ml, loosely filled with hydrophilic wicking material. Filter F1 consists of two 0.2 um hydrophobic filters in series and provides air to the system for filling, draining, and venting.

BIOREACTOR PLANS

The bioreactor system now under development for the immediate future is intended to support microcarrier culture.

Development efforts now underway consist of the following:

- Minimal media replacement and maximal water recycling
- Stirring/agitation techniques
- Automation
- Sensor development and application
- Maximizing product recovery
- Developing ways to culture specific, high producer cell fractions from the continuous flow electrophoresis system (CFES)

The following is a brief statement of our plans in each of the developmental areas.

- a. Minimal media - Use of a bioreactor or the CFES system in microgravity depends on the availability of water. Transport of that water to orbit is expensive, so media exchange or conditioning via dialysis is being explored to lessen that total transported water load. However, to de-

termine what in the media must be exchanged and how well that exchange occurs will require an analytical capability. A high pressure liquid chromatography system (HPLC) or equivalent system is proposed as the nucleus of that facility.

- b. Stirring/agitation - The purpose of stirring is to provide cell-media contact. Such contact can be achieved through the use of various blades or propellers submerged in the cell suspension. However, the use of mechanical devices can create severe turbulence and shear forces that may strip cells from their anchorage surfaces.
- c. Automation - In a space platform or even in a space station, crewmember time is at a premium; therefore, the bioreactor systems must be capable of limited unattended operation. Such systems depend on complex inter-relationships between the living cell and its environment. All the controlling factors of a cell culture are not known. Nevertheless, the system must be capable of self-monitoring and must have some decision-making capability.

We must first learn what needs to be controlled; second, we must learn the relationship between the controllable factors and, finally, we must learn how to apply electronic sensing and control techniques to manipulation of those factors.

- d. Sensor development - Most sensors (pH, O₂, CO₂, ROX, etc.) in use today are products of and for the laboratory. Sensor technology that is closest to bioreactor use is that used in the process control industry. In addition, some fermentor manufacturers market sensors; however, those sensors from both markets are generally inadequate for our use. They usually cannot be adequately sterilized, they may be too large, or they may leak electrolyte. Consequently, the sensors now in use in our bioreactor are regarded as marginal. Much additional sensor development must be undertaken before semi-automatic, computer controlled devices can be lifted into orbit.
- e. Product recovery - Bioreactors produce a relatively small amount of product in a large volume of media; therefore, methods must be investigated to extract as pure and as concentrated a product as possible. That product may then be routed to a CFES for purification, but only after significant concentration.

The method of choice to begin such a concentration regimen is ultrafiltration. A multi-staged filtration system composed of perhaps hollow fibers or thin channel apparatus is envisioned to accomplish the initial concentration.

- f. Specific fraction cultures - The CFES has the ability to separate specific cells from a large heterogenous population of cells. Those cells can be characterized as high or low producers of some specific product. If such high producers can be obtained in sufficient quantity, they can be grown in-mass in the bioreactor.

However, the mass rejuvenation of a CFES separated cell population has not yet been achieved. Additional CFES experience and time is necessary, and characterization of cell damage or changes due to electrophoresis must be conducted.

CONCLUSIONS

Two parallel lines of work are underway in the bioreactor laboratory. One of the efforts is devoted to the continued development and utilization of a laboratory research system. That system's design is intended to be fluid and dynamic. The sole purpose of such a device is to allow testing and development of equipment concepts and procedures. Some of the results of those processes have been discussed in this paper.

A second effort is designed to produce a "flight-like" bioreactor contained in a double middeck locker. The result of that effort has been to freeze a particular bioreactor design in order to allow fabrication of the custom parts. We expect the system to be ready for flight in early 1988. However, continued use of the laboratory system will lead to improvements in the space bioreactor. Those improvements can only be integrated after the initial flight series.

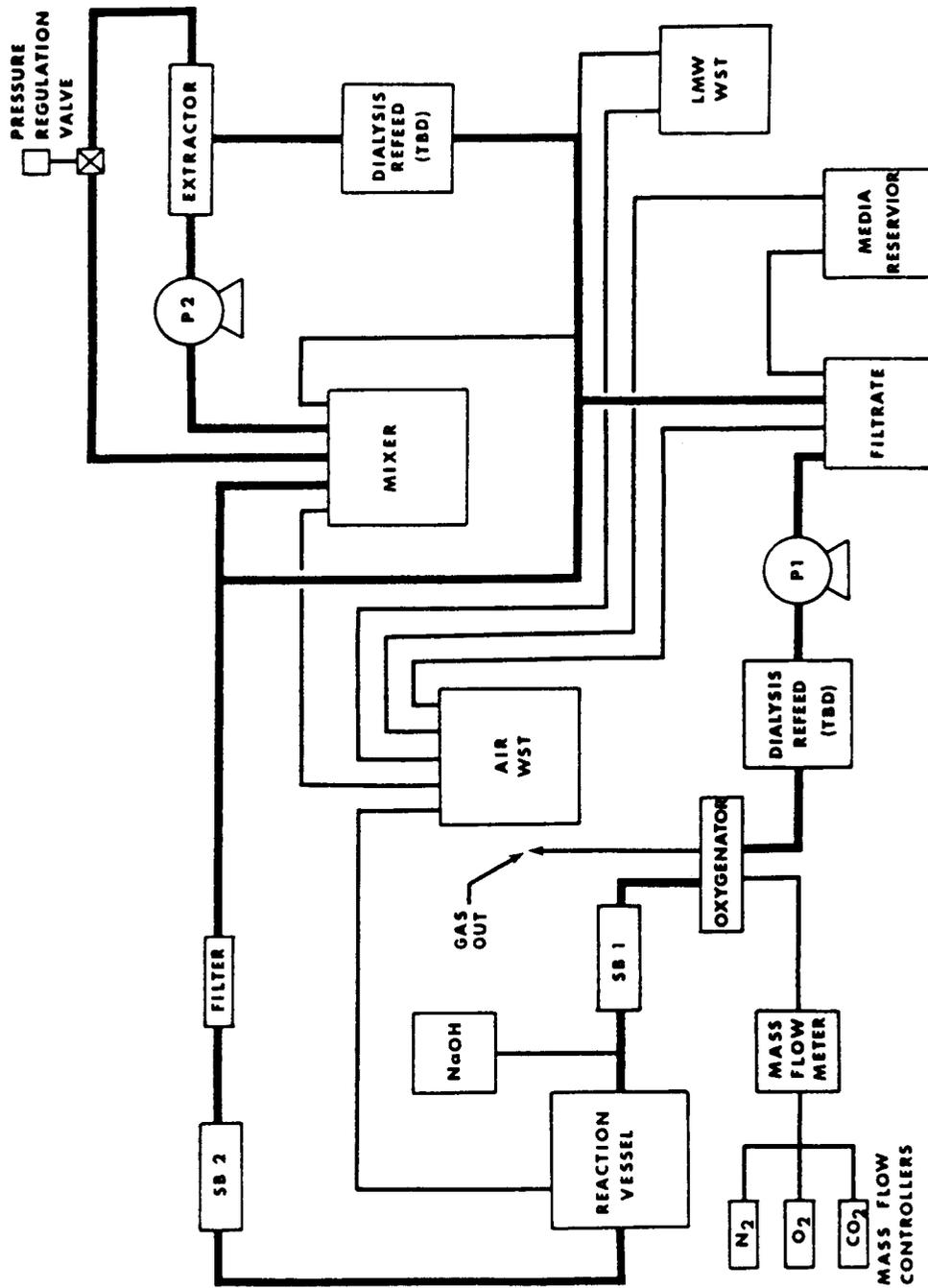


Figure 3-1.- Bioreactor block diagram.

COMPUTER CONTROL OF A MICROGRAVITY
MAMMALIAN CELL BIOREACTOR

William A. Hall, E.E.
Process Control Engineer
Technology Incorporated

INTRODUCTION

Our bioreactor is an electro-mechanical cell growth system cell requiring rigorous control of slowly changing parameters, many of which are so dynamically interactive that computer control is a necessity. The process control computer will have two main functions. First, it will provide continuous environmental control utilizing low signal level transducers as inputs and high powered control devices such as solenoids and motors as outputs. Secondly, it will provide continuous environmental monitoring including mass data storage and periodic data dumps to a supervisory computer.

The supervisory computer will be evolved with data analysis, plotting, and system parameters trend prediction.

This paper outlines the initial steps taken in developing a completely menu driven and totally automated computer control system for the bioreactor.

PROCESS CONTROL REQUIREMENTS

General Approach

The initial step in developing the process control system is to supply sufficient generalized control to determine individual parameter interaction. The data thus obtained will be used to develop the more sophisticated control loops. The requirements for the process control loops are divided into three major categories: dynamic, steady state, and/or on/off control. Each of these loops are described by the four following criteria:

- Limits - defines the maximum and minimum range of operation of the control loop
- Perturbations - sources of change or interferences to a particular control loop
- Control element - sensor used in feedback loop
- Effector - device acted on to provide control

Dynamic Control Loop

The dynamic control loops regulate pH, temperature, and gases, which require multilevel, interactive, control actions for optimal operating conditions. For example, any changes in oxygen flow rate or pressure will result in an equal change in dissolved CO₂ concentration followed by a shift in pH. This complicated interweaving of individual parameters and the long delay times for recorded responses, mean classical control approaches such as PID loops may not be adequate or reliable enough to maintain the desired degree of control. Moreover, the well known problem of biochemical sensor drift must be taken into account. Initial testing of the system is necessary in order to establish the intensity of parameter interaction in relation to the amount of transducer signal drift before any reliable interactive controls can be developed.

Steady State Control Loop

The steady state control loops regulate fluid flow rates and the reaction vessel stirrer motor's rpm. These loops maintain the flow rates and rpm at a set operating point independent of on-going system changes. The operator may change these fixed rates by a menu entry on the computer/operator display device.

On/Off Control Loop

The on/off control loops regulate pressure by activating a relay or solenoid based on the status of a fixed setpoint.

DEVELOPMENTAL REQUIREMENTS

General Developmental Requirements

Developmental flexibility in meeting time schedules and accommodating alterations in the bioreactor fluid system has resulted in four additional requirements.

a. Input/output requirements

The process control computer must be able to interface with a variety of analog inputs and provide a wide range of analog outputs. At present, these include 0-5 V dc (input and output), digital (input and output),

4-20 mA (input), and 0-20 V (output). In addition, the process controller must be able to communicate and exchange information with a separate supervisory computer.

b. Power/size requirements

The process controller must fit within a NASA Shuttle storage locker and consume no more than 80 watts. The maximum line wattage allowed on one line in the Shuttle is 125 watts. The Shuttle has three such lines. If a project requires two such lines, then that leaves one line for all the other projects and the crew. The same reasoning applies to the size limitation. As a general rule, the larger and more energy hungry a project is, the more difficult it is to get it into the Shuttle.

c. Programming requirements

The process controller must utilize a programming language which is compatible with a dynamically changing system and is easily understood by other members of the team. The programming language used during development must be usable able completion of the system or replaceable at this point without changing any electronic or fluid hardware. In the early stages, significant progress is expected in a short period of time. As an example, assembly language had initially been considered because it is one of the fastest and most compact of all the languages, but it is very difficult and time consuming to make large changes to programs in this language. An army of programmers would be needed to keep up with the fast research pace, and when all was said and done, only the programmers would be able to understand what the control program was really doing.

d. Support reliability requirements

The process controller must be supported by the manufacturer in terms of required modification and I/O boards. The point here is not to purchase a computer system whose manufacturer will out go out of business next month or who is unwilling to accept contract work for certain necessary modifications of sections needed to meet flight specifications. As an example, as the process control computer will be operated off of Shuttle dc power, a commercial process controller's ac power supply will be replaced. In some commercial process controllers, this would require a modification of the basic operating system software. Due to the complexity of some of the process controllers and the fact that the source code for the operating system is not provided to the users, only the manufacturer can make these changes.

Market Research

Guided by the above requirements, a comprehensive market research for a process controller was undertaken. The survey showed two types of process control units generally suitable for our application. The results of the survey are broken down into two major categories.

a. Commercially pre-packaged industrial process controllers

1. Advantages

The first group are the large industrial process controllers normally seen at chemical and oil refinery plants. These systems are generally very fast and can do just about anything. They have their own programming language and a set number of I/O boards (usually 6-10 different boards).

2. Disadvantages

The major disadvantage of this line of process controllers is that all analog computer interfacing must be made through one of these 6-10 different boards. For special input needs to the computer not supported by one of the 6-10 cards, the user must build special interface boards. This can easily lead to a significant increase in hardware. Another disadvantage most of these units have is that they are very large and power hungry. The few controllers which fall within initial size limitations (1 NASA Shuttle storage box) have other disadvantages. The customer must use the programming language supplied with the system. If this is insufficient to support the final version of the bioreactor, there is no way to change to another language. The customer is limited to a single vendor for both hardware supplies and support. And finally, the amount of programming space is inadequate. Most of these systems were designed for simple short control operations with a maximum of 40K bytes of programming space and no mass storage of data capability.

b. Bus level card process controller

1. General

These process controllers consist of individual computer boards which the user selects to make up the computer. Basically, the user builds his own computer from the board up. The actual central processing units can range from memory mapped or I/O mapped 8-bit microprocessors to the full 16 bits.

2. Advantages

There are several types of card level bus structures. The STD card system is one of the smallest and best supported of all the card systems. There are over 300 different manufacturers of STD cards and a variety of programming languages to choose from. In addition, the user can design and build his own interface boards and integrate them directly into the system without going through any other interface board. Mass memory storage is available in many different forms including bubble storage.

3. Disadvantages

The disadvantage of the 16-bit computer boards is their speed. Most STD cards on the market can handle up to a 4 MHz clock frequency, but the 16-bit boards operate at a much higher rate. The result is a limitation of variety of support I/O boards which can be utilized because the 16-bit CPU wants information faster than the support boards can deliver. This especially tends to be the case for "smart" support I/O boards which have their own microprocessor (i.e., smart analog input cards, etc.).

On the other hand, the 8-bit microprocessors can only address up to 64K bytes of memory cells. Since it takes 4 bytes of memory to store a real number, programming space becomes a real issue. In addition, a card level system requires the user to write the software drivers that will integrate the individual boards into a working system. This is an advantage in that the user can design his own boards and integrate them directly into the system, but is a disadvantage in that more programming space must be utilized leaving less for the actual control system.

Computer Selection Based on Survey

After an extensive market research and a consideration of all requirements, an 8-bit Z80 orientated STD card system with a multitasking basic programming language was chosen as the process control computer. This bus structure has flown with success on past Shuttle missions.

The multitasking basic programming language is understood by all and easily modified. In addition, each task is a separate entity and can be executed at different intervals. Each analog input and output board has its own task, and one task is reserved for an expert system.

Computer Operating Configuration

The bioreactor process control computer will be involved with a variety of operations. As seen in figure 4-7, the computer will not only interface with the bioreactor by means of transducers and effectors, but will also interface to printers, display panels, mass data storage devices, and other computers. The computer will utilize its sensors and effectors to control the bioreactor and periodically send formatted data to the printer, display terminal, and bubble memory card. Data will also be dumped both periodically and on-demand to the supervisory computer. When assistance is necessary, the computer will be able to telephone a control operator and transmit data and receive instructions. The computer will also provide a broad real-time view of the status of the bioreactor by means of the LED display board.

Computer Board Layout

The bioreactor process control computer, as seen in figure 8, consists of 12 cards. The CPU, Ram/Rom, serial, and modified CPU cards make up a functional computer. The rest of the cards are the computer's interface to the real analog world. Each interface card serves a unique function.

Analog Input Cards

Each of the 8-channel, 12-bit resolution, analog input cards has the ability to interface to a variety of signal inputs. This unique feature is due to the onboard computer located on each card which can be software programmed to interpret a variety of signals such as thermal couples, pressure transducers, or a variety of voltage ranges such a millivolts or volts.

Analog Output Cards

Each of the 4-channel, 12-bit resolution, analog output channels is capable of delivery up to 10 V in steps of .01 volts. These cards must be interfaced with off-board power drivers for high power devices.

Digital Input/Output Card

The digital I/O card consists of 64 channels which can operate as either TTL input or TTL output. The card will be used to detect switch closures, drive the LED display panel, and control opto-isolated relays.

Motor Control Card

The motor control card will be used to control stepper motors. If high powered steppers are required, then an external driver board will also be needed.

CONCLUSION

Although standard analog control would suffice for the simple control requirements, an industrial grade process control computer is required for the more involved processes normally requiring a great deal of analog circuitry and real-time human intervention of highly skilled personnel. Figure 4-1 outlines the control setup and requirements of the bioreactor as it now stands.

The 8-bit STD bus structure is adequate for an application consisting of slow changing parameters. However, if several PID type control loops are required in the end product, then these functions will need to be implemented a custom designed single board, STD control board which communicates with the main process controller. In this arrangement, the process controller passes parameters to the control boards, but is not involved with the continuous detail level control.

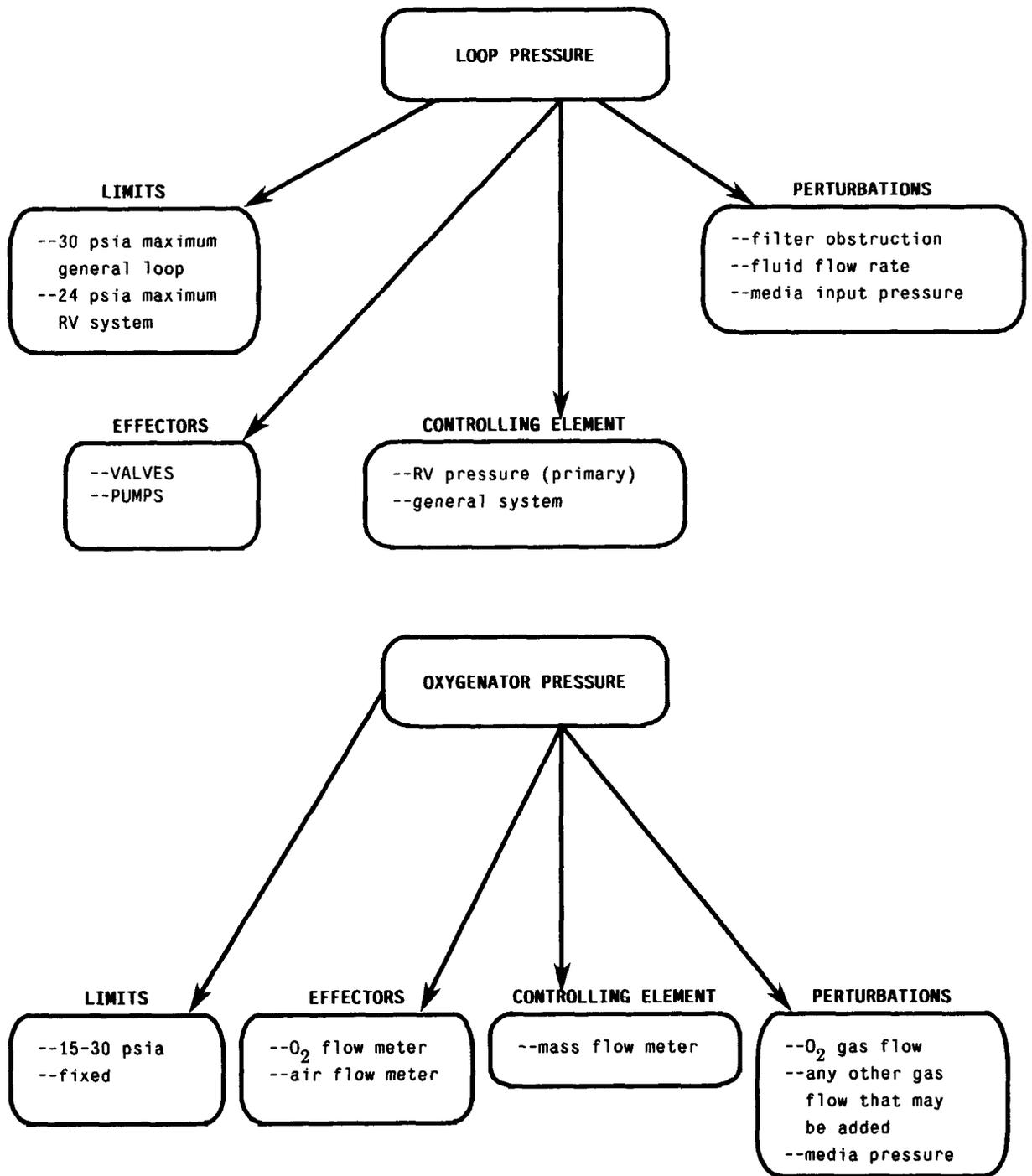


Figure 4-1.- System control requirements.

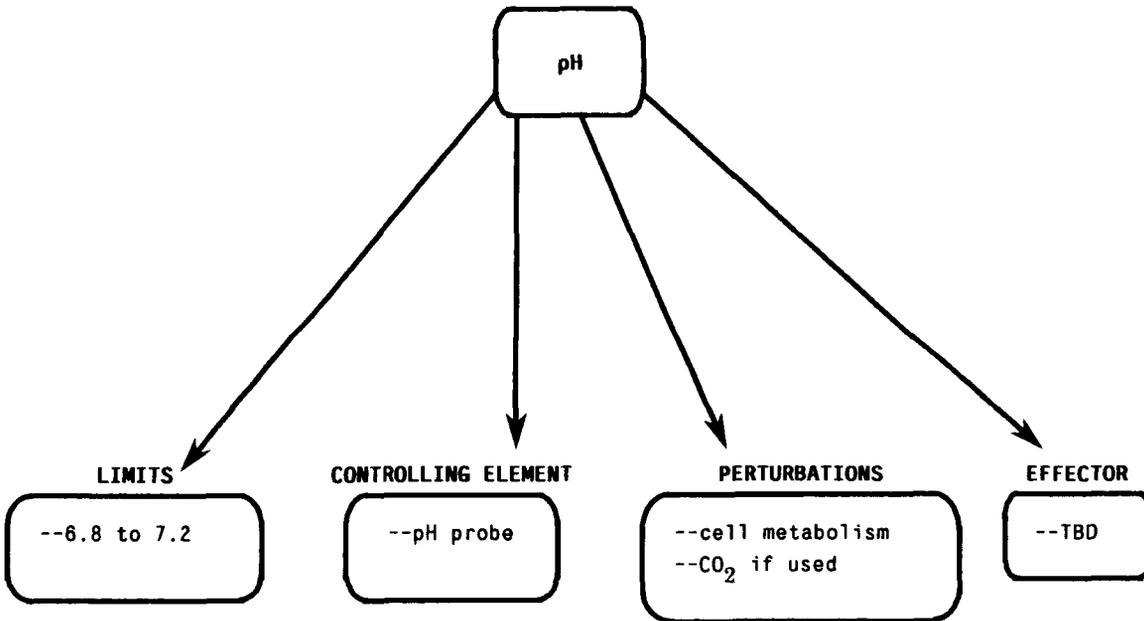
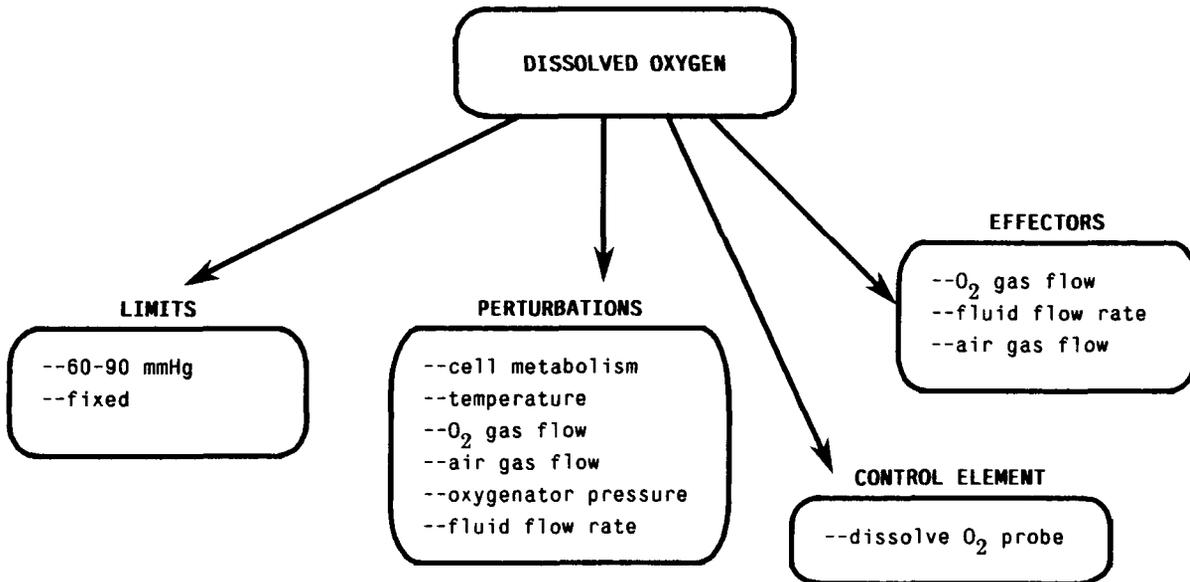


Figure 4-2.- System control requirements (continued).

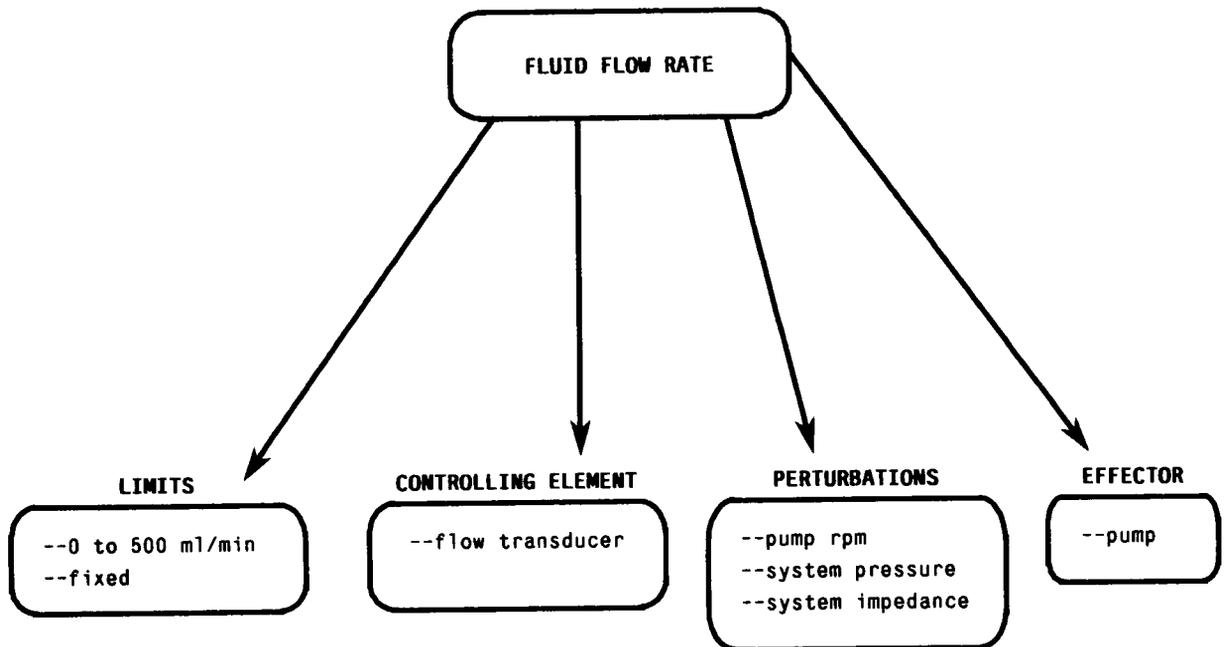
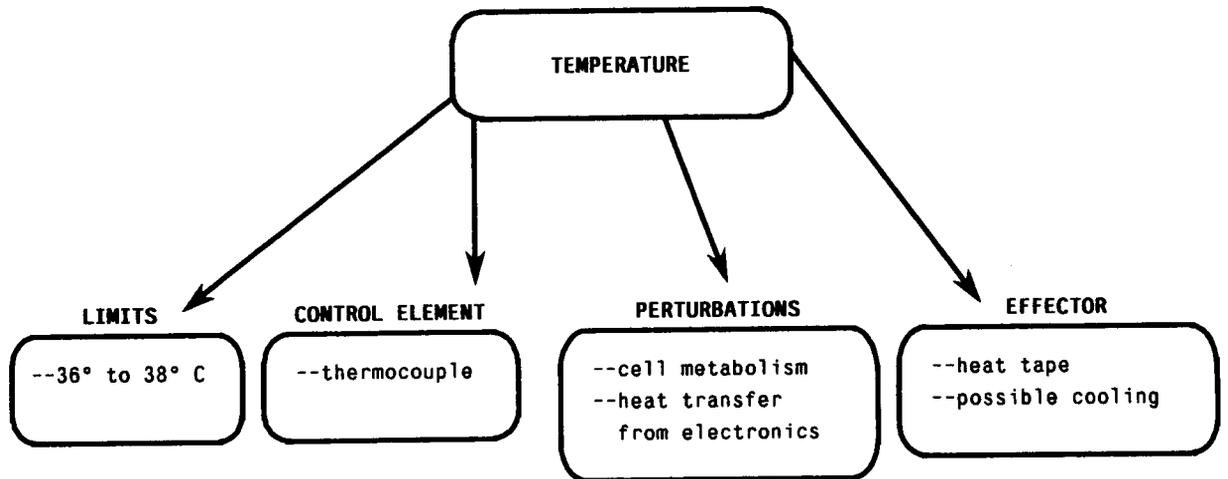


Figure 4-3.- System control requirements (concluded).

HARDWARE REQUIREMENTS

ANALOG I/O
DIGITAL I/O
FREQUENCY INPUTS
4-20 mA INPUTS
RS232
INTERFACABLE TO HIGH POWER DEVICES
--MOTORS
--HEAT TAPES

FLEXIBILITY REQUIREMENTS

--NUMBER OF BOARDS
--MULTIVENDOR

SOFTWARE REQUIREMENTS

--LANGUAGE EASILY UNDERSTOOD BY ALL
--LANGUAGE THAT EASILY HANDLES FREQUENT CHANGES
--NEED ABILITY TO CHANGE LANGUAGES IF NECESSARY

Figure 4-4.- Functional requirements.

LARGE INDUSTRIAL SYSTEMS

STD BUS

ADVANTAGES

--built-in language
--drivers provided
--monitor power

--choose a language
--abundance of vendors
--complete control of bus
--bus flow several times
--being strongly considered as
generic computer for NASA?

DISADVANTAGES

--must use their language
--cannot modify or add new drivers
--single vendor---few boards
--power hungry

--write own drivers
--choose a language

Figure 4-5.- Market research.

	µMAC5000	Fluke	Burr Brown	Std Buss
I.				
Inputs				
1. Transducers	yes	yes	yes	yes
2. Keyboard	no	no	no	yes
Outputs				
1. Transducers	yes	yes	yes	yes
2. Motors	yes	yes	yes	yes
3. Solenoids	relay	relay	relay	direct
4. Display	no	no	no	yes
5. Heat Tape	yes	yes	yes	yes
Cooling and Power				
1. 100 watt limit	yes	no	yes	yes
2. Cooling fans	yes	yes	yes	yes
Size				
1. Computer-locker	$\frac{3}{4}$	1	$\frac{1}{2}$	$\frac{1}{4}$
2. Entire system	2	>2	$1\frac{3}{4}$	$1\frac{1}{4}$
II. SOFTWARE				
1. 32k of Eprom?	yes	no	yes	yes
2. Data logging?	no	no	no	yes
3. Communication?	yes	yes	yes	yes

Figure 4-6.- Bioreactor computer comparisons.

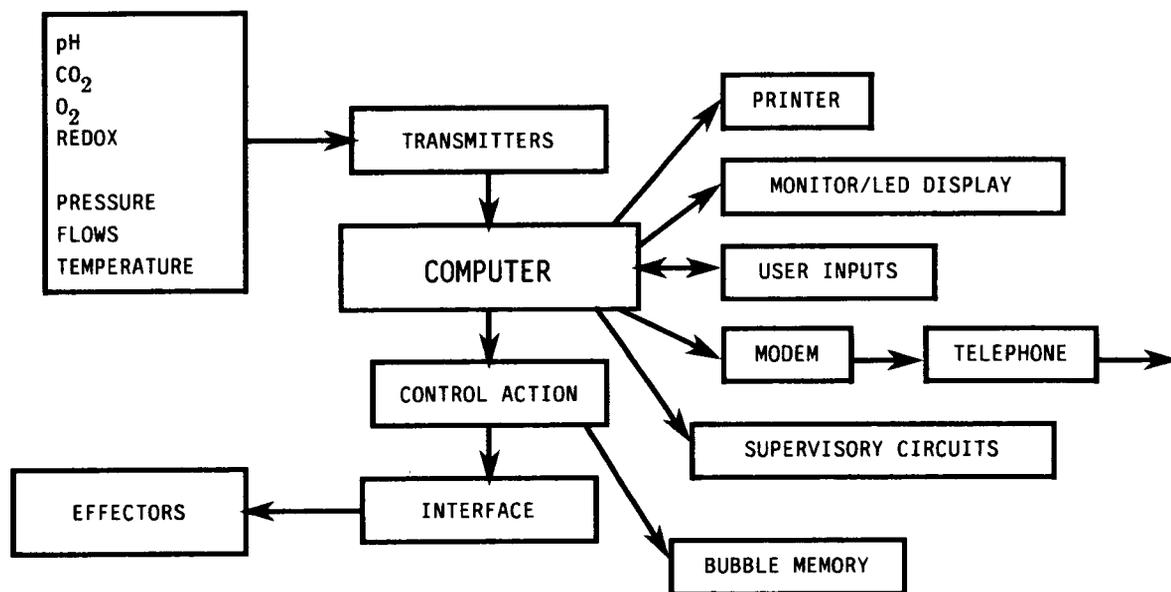


Figure 4-7.- Bioreactor process control computer interfaces.

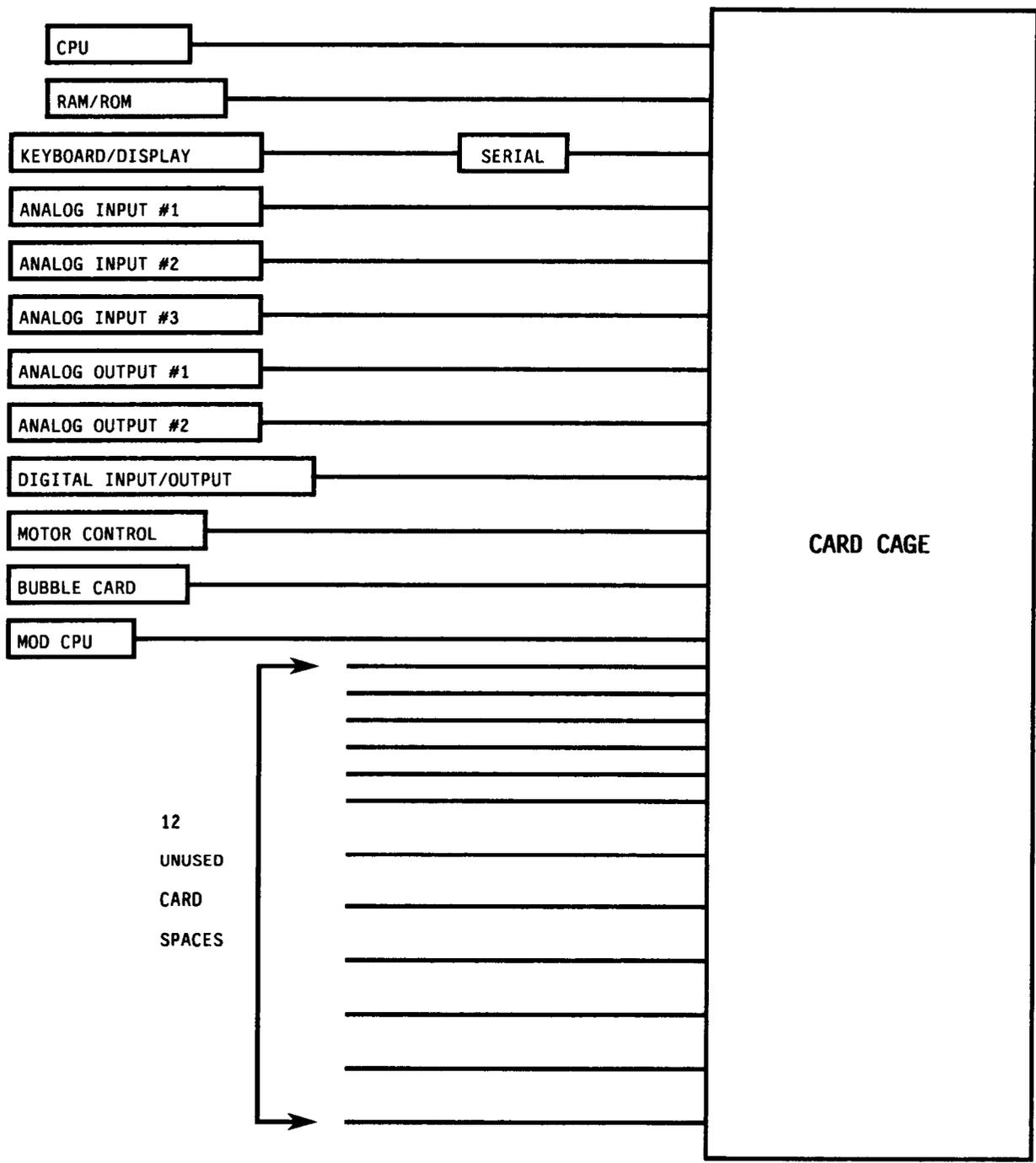


Figure 4-8.- The process controller.

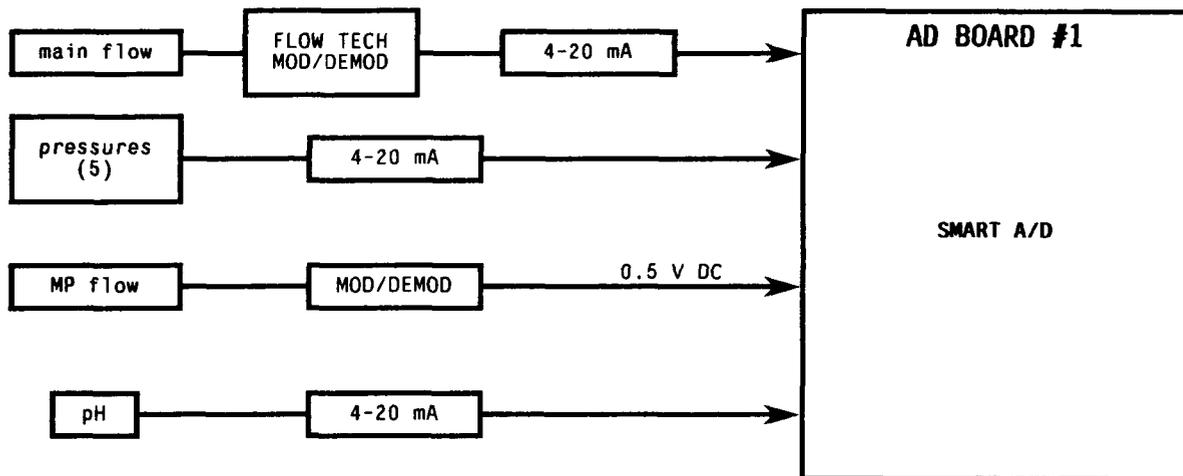


Figure 4-9.- The process controller (continued).

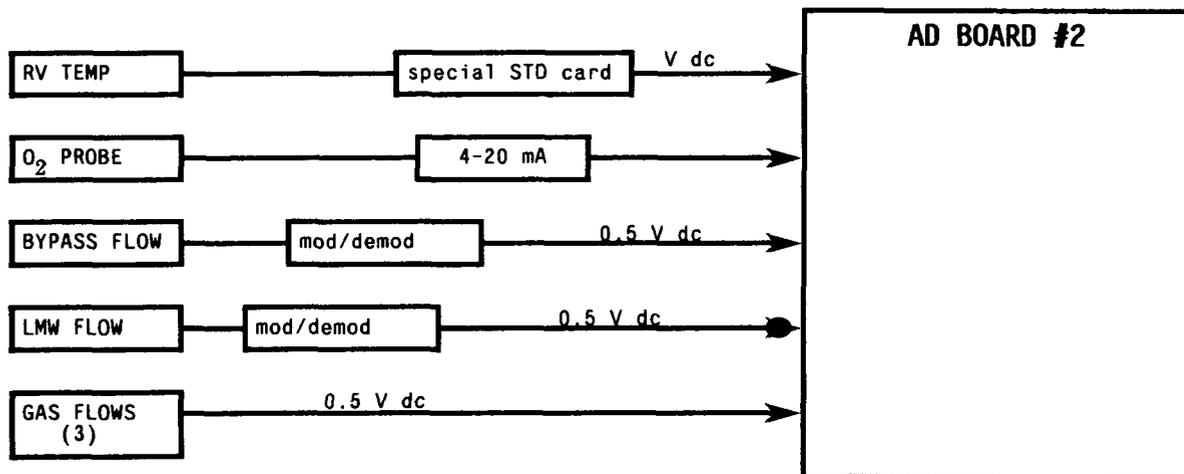


Figure 4-10.- The process controller (continued).

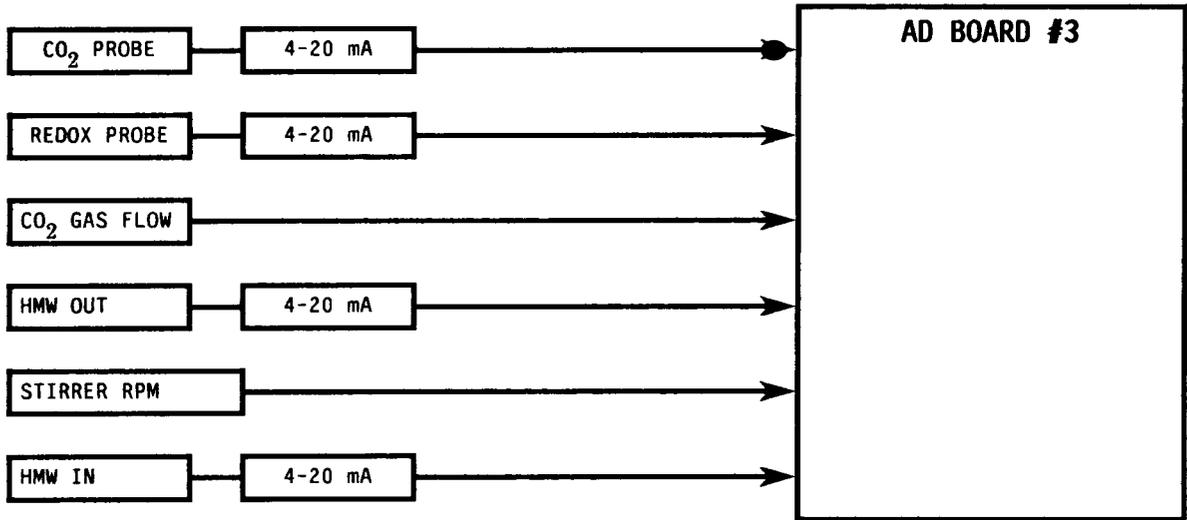


Figure 4-11.- The process controller (continued).

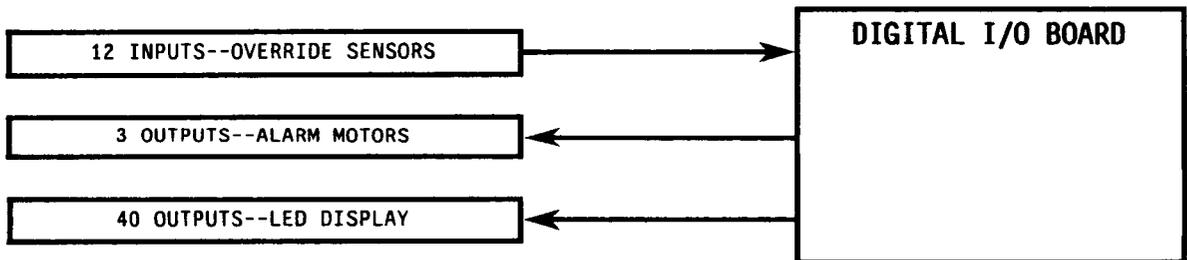


Figure 4-12.- The process controller (concluded).

SHEAR AND MIXING EFFECTS ON CELLS IN AGITATED
MICROCARRIER TISSUE CULTURE REACTORS

Robert S. Cherry and E. Terry Papoutsakis
Department of Chemical Engineering
Rice University, Houston, Texas

ABSTRACT

Tissue cells are known to be sensitive to mechanical stresses imposed on them by agitation in bioreactors. The amount of agitation provided in a microcarrier or suspension bioreactor should be only enough to provide effective homogeneity. Three distinct flow regions can be identified in the reactor: bulk turbulent flow, bulk laminar flow, and boundary-layer flows. Possible mechanisms of cell damage are examined by analyzing the motion of microcarriers or free cells relative to the surrounding fluid, to each other, and to moving or stationary solid surfaces. The primary mechanisms of cell damage appear to result from (1) direct interaction between microcarriers and turbulent eddies; (2) collisions between microcarriers in turbulent flow; and (3) collisions against the impeller or other stationary surfaces. If the smallest eddies of turbulent flow are of the same size as the microcarrier beads, they may cause high shear stresses on the cells. Eddies the size of the average interbead spacing may cause bead-bead collisions which damage cells. The severity of the collisions increases when the eddies are also of the same size as the beads. Bead size and the interbead distance are virtually equal in typical microcarrier suspensions. Impeller collisions occur when the beads cannot avoid the impeller leading edge as it advances through the liquid. The implications of the results of this analysis on the design and operation of tissue culture bioreactors are also discussed.

INTRODUCTION

Tissue cells, lacking a cell wall and not being evolutionary adapted to life exposed to a free-flowing liquid phase, are more sensitive to hydrodynamic forces in their environment than are fungi or bacteria. The particular problem usually cited in tissue culture work is shear from the agitator used to suspend the cells.¹⁻³ Various effects of shear have been reported. Most deal with cell viability,⁴⁻⁸ while some show increased production rates of some excreted products.^{8,9}

Shear has many manifestations within a stirred vessel contained suspended solids, not all of which would be expected to be harmful. This paper will consider the mechanisms by which hydrodynamic forces can affect cells in agitated cell culture reactors, and specifically microcarrier systems.

The effects of bulk liquid turbulence, boundary layers and shear fields, and collisions will each be considered. The results should be useful for rational reactor design and scale-up to any size.

PURPOSES OF AGITATION

Agitation of a cell culture reactor is required to keep the microcarriers from settling out and to assure a homogeneous environment for cell growth. In bacterial fermentations agitation is also used to control the amount of dissolved oxygen by affecting the oxygen transfer rate from the sparged gas into the liquid. In tissue cultures sparging may cause cell lysis and foaming, so other oxygenation systems are often used that diffuse oxygen through a tube or membrane or else oxygenate and recirculate medium from which the cells have been separated.^{1,2} Agitation is not critical to oxygenation with those systems,¹⁰ so settling and homogeneity will be considered individually to determine how much agitation is minimally required.

The first item, preventing settling, requires a negligible fluid velocity in the bulk phase. Assuming microcarrier and liquid properties as in table 5-1, Stokes' law gives a terminal velocity v_t of 0.053 cm/s. The maximum shear stress on the bead surface that results from this velocity is ~ 0.1 dyne/cm², well below the 10 dyne/cm² that starts to damage kidney cells.⁸

Maintaining homogeneity by minimizing variations throughout the reactor of dissolved oxygen and other nutrient concentrations or temperature is the primary reason for agitating tissue culture reactors. There will inevitably be local variations, for example higher oxygen concentration near the oxygen source, or slightly different temperatures at the wall of a jacketed reactor. We can approximate the average liquid velocity needed to give effective homogeneity by requiring that the cells move through these areas of different conditions in an amount of time that is small compared to their metabolic response time. Although there is apparently no data published for tissue cells, several references for bacteria^{11,12} and yeast¹³ suggest that cells do not respond to transients of two seconds or less.

Saying that a one liter cell culture reactor has a characteristic dimension of 10 cm, the minimum liquid velocity needed is on the order of 10 cm/(2 s), or 5 cm/s. This is about one hundred times the settling velocity of the microcarriers, so mixing of the liquid that is sufficient to keep cells from lingering in areas of locally different conditions will be more than enough to keep the microcarriers or free cells from settling under gravity's influence.

In addition, mixing and its associated mechanical stresses may be beneficial in enhancing growth and/or product formation due to the physiological effect

of fluid shear stresses.^{8,9} In that case, the mixing or agitation of the bioreactor should be designed to provide the spectrum of stresses that gives the optimal cellular response.

BULK LIQUID TURBULENCE EFFECTS

The structure of isotropic turbulence was originally formulated by Kolmogorov in 1941.¹⁴ The kinetic energy of the velocity fluctuations in turbulent flow is passed from larger eddies to smaller ones with minimal dissipation until, in the smallest eddies, viscous losses degrade the kinetic energy to heat or in this case possibly to mechanical work in physically damaging cells.

The liquid flow in a typical stirred reactor is at least locally turbulent because of the high impeller tip speed and the various probes, thermowells, and sampling tubes that act as baffles. If the scale of the smallest turbulence is sufficiently larger than the microcarriers, the beads just follow the local flow pattern (fig. 5-1a) and move at the local liquid velocity.¹⁵

Turbulent eddies of the same size as a microcarrier, however, may effect cell performance in several possible ways. A single eddy cannot engulf the bead and can only act on part of the surface, causing the bead to rotate and generating a cyclic shear stress. Frangos et al.,⁹ found a 1 Hz shear variation to have a significant positive effect on prostacyclin production. The general effects on cells of other frequencies are unknown. However, in one case¹⁶ fibroblasts gave up to a 30 times increase in specific interferon production when grown on microcarriers in spinner bottles versus on the walls of roller bottles under identical conditions, although no explanation for the increase was offered.

Alternatively, several eddies the size of the microcarrier could interact with it simultaneously. If their actions are opposed to one another, the eddies cause a greater shear stress against the part of the microcarrier nearest them (fig. 5-1b) since the bead cannot rotate to cancel each of the shear forces on it.

Turbulent eddies of the same size scale as the microcarrier separation may also cause cell damage by promoting bead-bead collisions. Eddies much larger than the bead spacing can move groups of beads without causing large relative velocities between them. It is easily conceivable that eddies the size of the interbead spacing could accelerate one bead without disturbing another nearby (fig. 5-1c). The two beads then have a significant relative velocity and a finite chance of collision.

The collision frequency per unit volume N_c for suspended particles is of the order¹⁵

$$N_c = 0 \left[\frac{v_{b,r} \alpha^2}{d^4} \right]$$

where $v_{b,r}$ is the root mean square relative velocity between neighboring particles, α is the volume fraction of beads and d is bead diameter.

Substituting for α as a function of bead spacing, d_s , and setting the relative velocity equal to that of eddies the size of the interbead spacing ($v_{b,r} = v/d_s$ by Kolmogorov's theory),^{14,17,18} and since for typical conditions the bead spacing is approximately equal to the bead diameter d ,

$$N_c = 0 \left[\frac{vk^{7/3}}{d^{8/3}} \right] s^{-1} cm^{-3}$$

where k is proportional to the required bead surface area per reactor volume. Thus, the collision frequency is strongly dependent on the particle diameter when the smallest eddies are the size of the bead spacing. For typical conditions, $k = 2.4 \text{ cm}^{-1}$ and $N_c \sim 4,000 \text{ collisions/s-cm}^3$, or roughly one collision per bead every five seconds.

The severity of collisions (SC), defined as the energy (E_c) times the frequency (N_c) of collision, will be of the order of

$$SC = 0 \left[\left(m v_{b,r}^2 \right) N_c \right]$$

or

$$SC = 0 \left[\frac{\rho_b n v^3 k^{7/3}}{6d^{5/3}} \right]$$

where m is the mass of an individual bead. The effect of severity of collision on the cells may be hard to quantitate because the cellular responses and severity are unlikely to be linear - if a certain blow kills the cell, hitting it twice as hard does not make it twice as dead - so the net effect is uncertain. It could conceivably be in either direction depending on the relative sensitivity to the frequency and energy terms.

The collisions between beads can have a variety of effects on the cells covering the beads. A head-on collision flattens the cells at the point of collision, possibly rupturing them depending on the energy of collision and the elasticity of the cells. As the collision becomes more and more off-center, the cells in contact between the two beads see less compression but a larger component of shear force, which will in turn depend on the coefficient of friction of two cells sliding over one another, the cells feel only a shear force. The gross effect of this may be either cell rupture or detachment from the bead surface. The physiological effects of nonfatal compression or mechanical shearing are not known. The analysis of collision is further complicated by any rotation the beads may have, which would in general contribute an additional shearing component to the force of the collision.

The smallest eddy size may also be calculated if the impeller geometry and operating conditions are known. There exists relationships (fig. 5-2) that relate dimensionless power consumption N_P to impeller Reynolds number N_{Re} and specific turbulent energy dissipation ϵ , and ϵ to eddy size η :

$$N_{Re} = \frac{d_i^2 n \rho_f}{\mu}$$

$$N_P = \frac{P_g}{\rho_f n^3 d_i^5}$$

$$\epsilon = \frac{P_g}{\rho_f V} = \frac{N_P n^3 d_i^5}{V}$$

$$\eta = \left(\frac{v^3}{\epsilon} \right)^{1/4}$$

where P is power consumption by the impeller, n is impeller speed in revolutions per unit time, d_i is impeller diameter, and V is the agitated liquid volume.¹⁹ Using typical values, the predicted eddy size is 0.012 cm, which compares with a microcarrier diameter of 0.015 cm and a typical bead spacing of 0.018 cm.

To see the effect of some important reactor variables, the N_p expression for ε is substituted into the expression for eddy size η :

$$\eta = \left(\frac{v^3 V}{N_p n^3 d_i^5} \right)^{1/4}$$

Reactor volume V is fixed by production requirements. N_p varies in a relatively narrow range for reasonable values of N_{Re} so the $\frac{1}{4}$ power of it is ineffectual in significantly changing eddy size. The important factors to change eddy size are $v^{3/4}$, $n^{-3/4}$, and $d_i^{-5/4}$.

In summary then, cells on beads are most affected by turbulence of a size scale the same as the average bead spacing or bead diameter (causing collisions) or the bead diameter (causing rotation or high local shear on the bead surface). In a typical one liter reactor these dimensions are effectively the same, emphasizing the empirical significance of this eddy size. The turbulent eddies may be made larger, and cell damage presumably reduced, by increasing kinematic viscosity or reducing impeller diameter and speed. If the eddy size cannot be sufficiently increased, using a larger bead diameter may reduce the collision frequency, and may, depending on the behavior of the cells, improve the performance of the bioreactor.

BOUNDARY LAYER SHEAR FORCES

Relatively large areas of high shear rate are expected in the boundary layers around the solid objects submerged in the reactor. The moving impeller would be expected to have the highest velocity relative to the liquid, so we shall analyze it in detail to characterize the general effect of boundary layer shear forces on microcarriers. Much of this discussion can also be applied to the hydrodynamically similar case of the physically much larger shear fields expected in a non-turbulent, laminar flow reactor.

As a first approximation marine and angled flat impeller blades can be modelled as stationary flat plates with fluid moving over them. Boundary layer thickness and wall shear stress for both turbulent and laminar boundary layers are shown in figure 5-3. Up to 0.3 cm from the impeller leading edge the two types of flow give significantly different results, but this is also the area where the flat plate assumption of the calculations is least valid and the presence of a microcarrier bead causes the greatest disruption to the boundary layer. Past 1 cm, and over the majority of the blade, the results are similar: there is a boundary layer of 0.1 cm thickness (≈ 7 bead diameters) with a relatively low shear rate within it.

Within the boundary layer a number of effects may occur (table 5-2). Considering the simpler case of a laminar boundary layer, the bead will certainly try to follow the fluid motion which has components both parallel to and perpendicular to the blade surface. Particle motion parallel to the blade is a combined result of the particle's initial velocity and fluid drag. There is also an effect due to the presence of the solid impeller surface that slows the particle's motion.²⁰ This retardation is particularly important when the bead is within one radius of the surface. There are two other parallel forces,¹⁵ the Basset force, which arises from the work necessary to establish a new fluid flow pattern when the bead is accelerated rapidly, and the added mass effect, which accounts for the behavior of the displaced fluid. These terms are negligible over most of the impeller, and are of consequence only at the leading edge.

The fluid velocity which causes the drag force normal to the impeller is a consequence of boundary layer development in an incompressible fluid and is directed away from the impeller. On the upper surface of the blade, gravity opposes the drag force of this normal flow. As with parallel motion, near the wall the hydrodynamic effect of the fixed surface damps any vertical motion.

There is also a lift force derived from the velocity gradient in the boundary layer.²¹ This Saffman lift force is present only when the bead has a slip velocity relative to the fluid streamline that would pass through the sphere's center. It acts to move the bead towards the streamlines which most oppose the slip velocity, so for example, a bead moving faster than the local fluid tends to move down the velocity gradient. Near the impeller leading edge the bead will move over the impeller surface faster than the fluid because of its initial inertia, and the lift force will be toward the blade. Further back on the blade fluid drag will slow the bead and the effect of the nearby surface causes the bead to lag the fluid motion. This lag has been demonstrated by Einav and Lee,²² and the resulting lift force is away from the impeller. There is another lift force acting, from the Magnus effect on a sphere rotating in a constant velocity field. This force is superimposed on the Saffman force in this system. However, Saffman²¹ has shown that the Magnus force is negligible compared to the lift caused by the shear field.

The shear field in the boundary layer also causes the bead to rotate.^{23,24} A rotational rate on the order of 20 revolutions per second is predicted, similar to the 10 Hz predicted for turbulent rotation. With 250 s^{-1} as the average shear rate in the 0.1 cm boundary layer of the example system, the maximum shear stress on the bead due to this rotation is of the order $3_{\mu\text{yf}}$ or 5 dyne/cm^2 , a nondestructive level.⁸

Overall, in a laminar boundary layer the microcarrier bead appears well protected from damage. The particle tends to move away from the impeller

surface (except perhaps near the leading edge), it rotates at a moderate speed, and the cells on its surface do not see excessive shear stress. There are no bead-bead collisions either.²³ In a turbulent or separated boundary layer the same basic situation holds except for the additional presence of turbulent eddies (discussed under bulk turbulence). These create the possibility of bead impact against the impeller or other beads because of randomly oriented velocity fluctuations occurring in the boundary layer or intruding from the bulk liquid.

COLLISION DAMAGE

High velocity collisions of a microcarrier against the impeller or other parts of the reactor can occur when the blade advances through the fluid or the fluid flows around a fixed object (fig. 5-4). Microcarriers flowing on a streamline that passes within one particle radius of the surface will collide with the surface, a process called interception.²⁵ In addition, the microcarriers, being slightly more dense than the fluid, will not follow the fluid streamlines exactly. Inertia will tend to make the microcarrier travel in a straight line rather than flow around the object with the fluid, increasing the chance of collision. The deviation from the fluid streamline will be more severe where the streamlines are most curved, as is the case at the leading edge of the impeller blade. Using potential flow theory to model the streamlines and ignoring bead inertia, one may show that any bead vertically within one bead diameter of the streamline passing through the center of the cylinder used as the leading edge model will hit the impeller.

Considering the width of this collision window, its length (impeller blade length $d_i/2$ times the number of blades n_B) and the velocity of medium through this window, one may calculate that, on average, each microcarrier hits the impeller once each 220 seconds if the entire one liter reactor is well mixed. This is 1/40 the frequency of bead-bead collisions, but the energy of collision is 2500 times greater because of the higher relative velocity.

The collision rate is proportional to the agitator speed since inertial effects on the width of the collision window are not included in this calculation. In addition, the kinetic energy of the collision is much higher, increasing with bead mass and the square of impact velocity which is proportional to agitator tip speed. Combining these effects,

$$SC_i = N_{c,i} E_{c,i} = 0 \left[\frac{m n_B d_i dn}{2V} \right] 0 \left[\frac{m}{2} \left(\frac{3}{4} d_i \right)^2 \right] = 0 \left[\frac{3}{128} n^4 \frac{\rho_b n_B n^3 d_i^4 d^4}{V} \right]$$

Severity of collisions with the impeller is proportional to the cube of the agitator speed and the fourth power of impeller diameter and bead diameter. Since tip speed equals πnd_i , note that there is also a third power dependence on tip speed. However, as with bead-bead collisions in turbulence, the effect of collision severity (as defined here) on such things as cell viability or maximum cell density is certainly not linear, and may even have a minimum or maximum within the practical range of severity values.

The nature of the surface the cell covered bead hits will affect the amount of cell damage that results. A hard surface will concentrate the total collision force on one or two cells directly in contact with the surface, and will perhaps cause the bead to distort and disrupt cell attachment. An elastic impeller coating softer than the bead could both absorb some of the collision energy and distribute the remainder over a broader area on the bead, reducing the force that individual cells are subjected to.

Smoothness of the impeller surface is important too, to avoid spikes or sharp-edged holes or ruts that could cause damage during what might only have been a glancing impact. Such surface roughness would be significant at the scale of the individual cells' dimension - about 10 μm . Avoiding this potential problem requires a very smooth surface, suggesting that polishing of machined, cast, or welded impellers would be of benefit.

IMPLICATIONS FOR REACTOR DESIGN

Two effects stand out as likely causes of cell damage or poor performance in microcarrier tissue culture reactors: turbulence of a size scale comparable to the microcarriers or the spacing between them, and collisions with solid objects, particularly the impeller. The smallest eddies in a turbulent flow

are characterized by a length scale $\left(\frac{v^3}{\epsilon}\right)^{1/4}$. This size has been increased empirically by reducing ϵ , the local energy dissipation rate, through such design changes as eliminating baffles, using marine rather than paddle impellers, reducing agitator speed, and using hemispherical rather than flat reactor bottoms. Each of these reduces turbulence, hence ϵ , in some part in the reactor.

Further advances in increasing the scale of turbulence can be achieved by raising the fluid kinematic viscosity. Because the turbulence scale depends on $v^{3/4}$ compared to $\epsilon^{1/4}$, the effect should be much stronger. To minimize osmotic effects, high molecular weight polymers or gums are good candidates to add to the culture medium. High polymers are also known to reduce drag, and therefore agitator power consumption, further increasing eddy size. A beneficial effect of polymer addition on free-living human lymphoblastoid

cells has been reported,²⁶ although the effect was hypothesized to be mechanical protection of the cells by adsorbed polymer and possibly related to surface tension.

The size of the microcarrier beads should be optimized for each application. In systems where impeller collision is the primary source of damage, smaller beads have a lower collision frequency and a lower kinetic energy of collision. If bead-bead collision in turbulent eddies is the major damage mechanism, decreasing bead size lowers the collision energy, but raises the frequency. Depending on which factor is more important, the optimal bead size may be either smaller or larger.

Collision damage can be minimized by rational impeller design. The smallest impeller, in terms of both diameter and number of blades, that gives adequate mixing should be used. Streamlining the blade cross-section, and in particular rounding the leading edge, will reduce the number of collisions. As noted already, polishing any rough surface and applying an elastic coating would mitigate the effects of any collisions that do occur.

Recalling that mixing is needed primarily to prevent relatively stagnant zones from forming, it is desirable to establish a large scale circulation without extremes of velocity. This could be accomplished and impeller collision estimated by using externally recirculated liquid for mixing rather than using an agitator. The turbulence around the return jets must be considered, but could be controlled by limiting the jet velocity. The biggest problem is likely to be developing an effective way of separating the microcarriers from a relatively large flow of culture medium so that they would not be damaged in the circulation pump.

Alternatively, it is possible to utilize secondary flows generated by a very low speed agitator to provide reactor mixing,²⁷ although this may not be sufficient as reactor size increases. This may be the operating principle behind the successful use of soft spiral vanes for agitation.²

CONCLUSIONS

By analyzing the phenomena involved in agitation of microcarrier suspensions, harmful effects on cell cultures that have been attributed to "shear" are found to be better explained as effects of turbulence or collision. Changes meant to reduce shear have also reduced turbulence and collision, leading to improvements in the practice of cell culture. Other new approaches to reducing turbulence and collision are proposed. Studies to determine the response of cells to intermittent cyclic shear stress at frequencies in the range of 5 to 30 Hz are suggested.

ACKNOWLEDGMENT

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TABLE 5-1.- REPRESENTATIVE MICROCARRIER REACTOR SPECIFICATIONS

Liquid

Volume, V	1 liter
Density, ρ_f	1.0 g/cm ³
Viscosity, μ	0.007 cP

Microcarrier beads

Shape	Smooth spheres
Radius, R	75 μ m
Density, ρ_b	1.03 g/cm ³
Concentration, α	
- dry basis	5 g/liter
- hydrated	7 vol %

Impeller

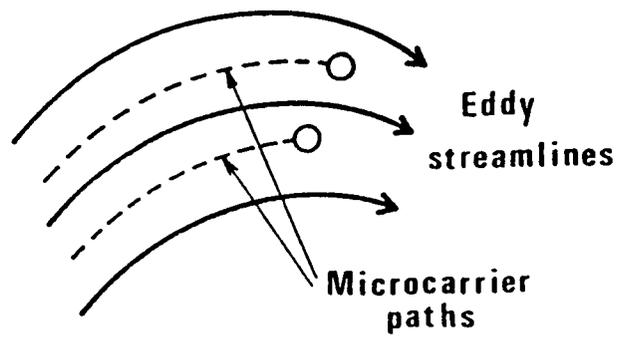
Configuration	4 rectangular blades at 45° angle
Diameter, d_i	8 cm
Blade width, w	3 cm
Leading edge radius, R_i	0.1 cm
Rotational speed, n	60 rpm
Tip speed, v	25 cm/s

TABLE 5-2.- BOUNDARY LAYER FORCES

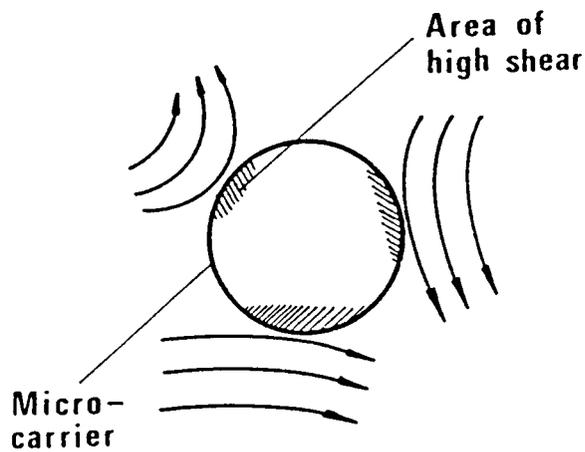
<u>Force</u>	<u>Resultant bead motion relative to surface</u>
Fluid drag	Parallel, normal, and/or rotational
Gravity and buoyancy	Normal
Effect of pressure gradients*	Parallel, and/or normal
Saffman lift force ²¹	Normal
Added mass effect** ¹⁵	Parallel
Basset force** ¹⁵	Parallel
Magnus force** ²¹	Normal

* Important only in turbulent boundary layers

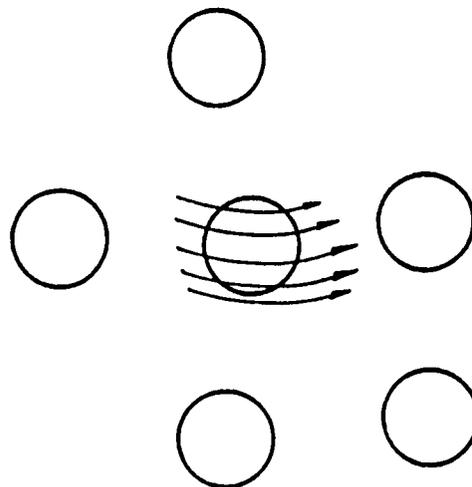
** Not important in this system



(a) Eddies much larger than the beads.



(b) Multiple eddies same size as bead.



(c) Eddy size same as interbead spacing.

Figure 5-1.- Bead-eddy interactions.

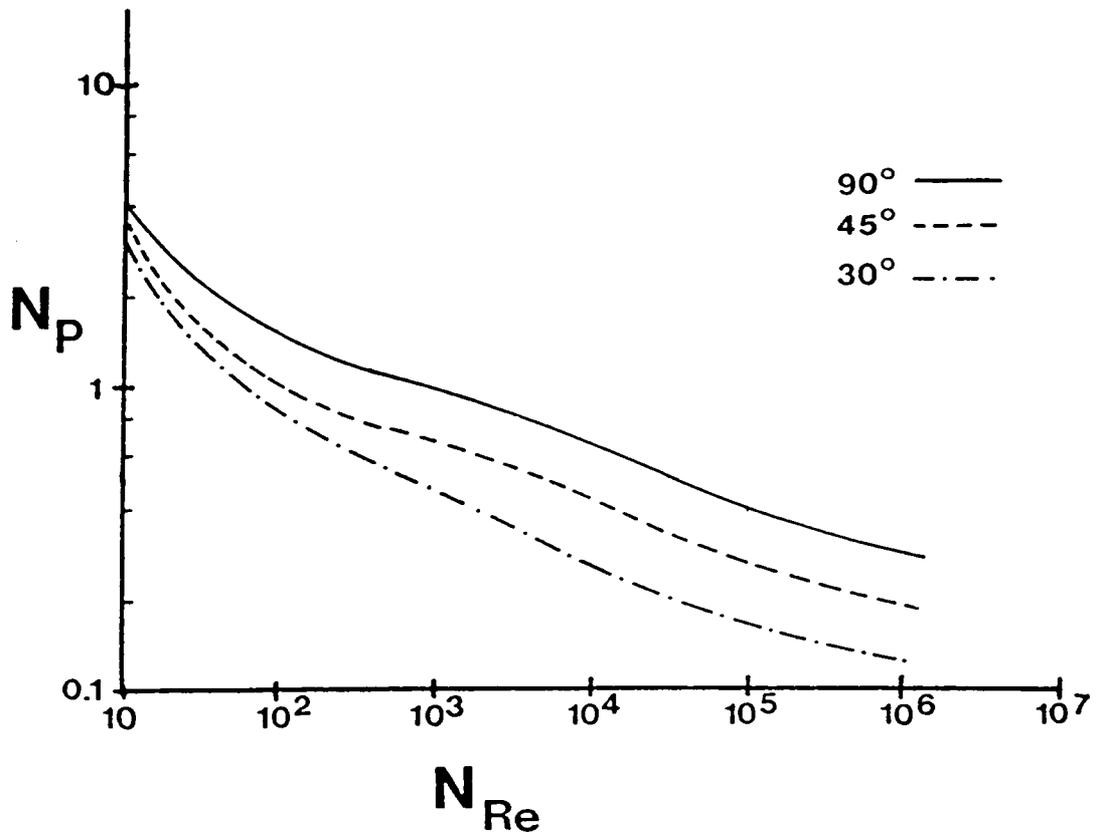


Figure 5-2.- Power number correlation for two-bladed impeller at various blade angles. Adapted from Nagata (fig. 1.21).¹⁸

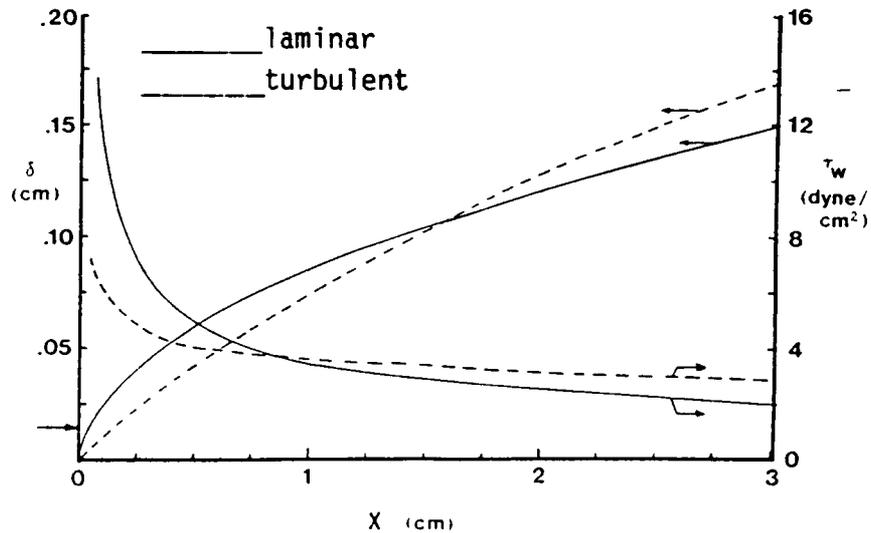


Figure 5-3.- Boundary layer thickness and wall shear stress on the impeller. Arrow indicates microcarrier diameter.

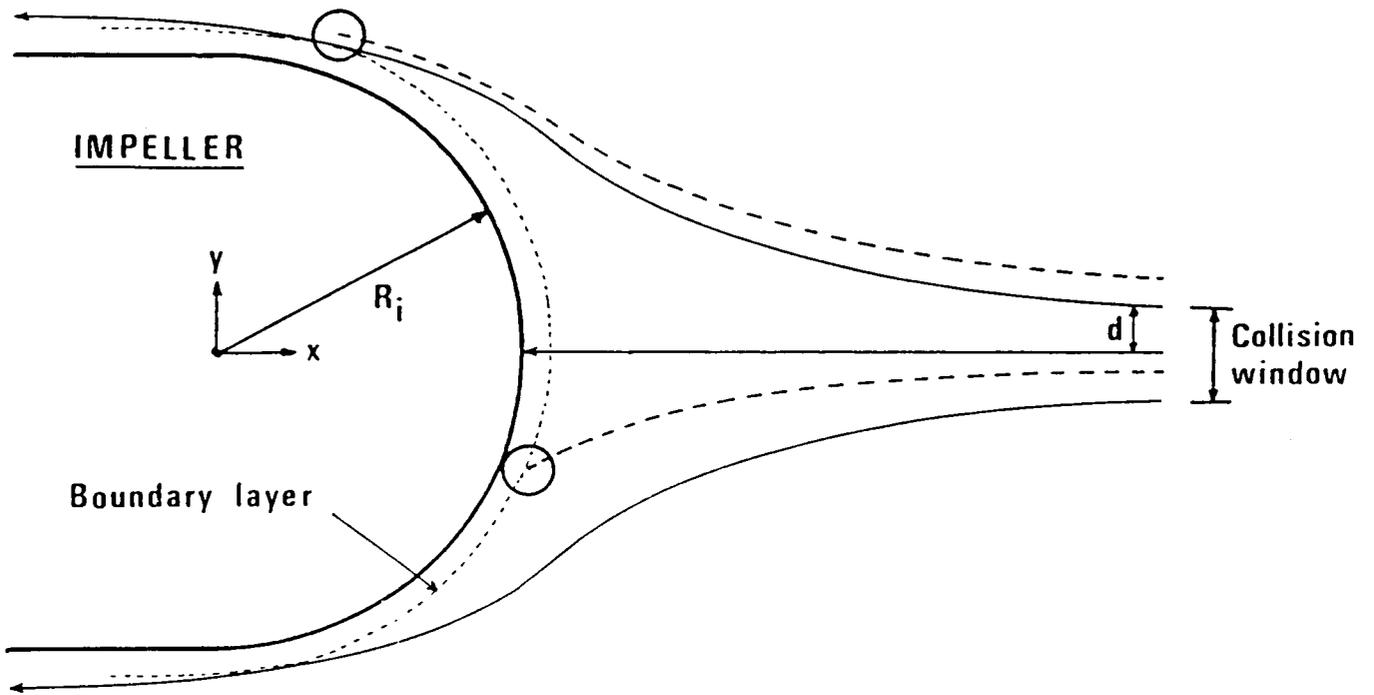


Figure 5-4.- Streamlines around impeller leading edge. Beads inside the collision window strike the impeller.

SOME PROCESS CONTROL/DESIGN CONSIDERATIONS IN THE
DEVELOPMENT OF A MICROGRAVITY MAMMALIAN CELL BIOREACTOR

Charles F. Goochee
Assistant Professor
Department of Chemical Engineering
University of Houston, Houston, Texas

INTRODUCTION

The purpose of this paper is to review some of the physical/metabolic factors which must be considered in the development of an operating strategy for a mammalian cell bioreactor. Particular emphasis will be placed on the dissolved oxygen and carbon dioxide requirements of growing mammalian epithelial cells. Literature reviews concerning the oxygen and carbon dioxide requirements of mammalian cells will be presented first. This will be followed by the presentation of a preliminary, dynamic model which encompasses the current features of the NASA bioreactor. This paper concludes with a discussion of the implications of this literature survey and modelling effort for the design and operation of the NASA bioreactor.

DISSOLVED OXYGEN AND EPITHELIAL CELL GROWTH: LITERATURE SURVEY

Few relevant literature articles are available concerning the oxygen utilization of cell cultures and the influence of the dissolved oxygen concentration on cell growth and product information. The principal focus of this limited literature has been the metabolism of fibroblast cell lines, such as WI-38. It is now generally recognized that the growth and plating efficiency of fibroblast cells are inhibited by dissolved oxygen concentrations corresponding to 140 to 160 mm Hg. The optimal dissolved oxygen concentration is of the order of 40 mm Hg or less, which corresponds to the physiological level of dissolved oxygen.^{1,10,11} Balin et al. (1984) have noted that dissolved oxygen inhibition is particularly pronounced when fibroblast cells are grown against 20% oxygen at a low seeding density.

Published oxygen utilization rates (OUR) for fibroblast cell cultures vary over a broad range--from 0.06×10^{-12} to 0.5×10^{-12} moles of oxygen utilized per cell per hour.⁴ This variation may be partially due to a variation in the OUR between growing and confluent cells. Balin et al. (1976) noted that WI-38 cells in logarithmic growth utilized oxygen at a rate of 0.5×10^{-12} moles per cell per hour, whereas stationary phase WI-38 cells utilized oxygen at a rate of 0.2×10^{-12} . Variation in medium composition may also contribute

to the broad range of OUR values which have been observed, but no literature references were found which addressed this question.

The literature concerning the effect of dissolved oxygen concentration on the growth of epithelial cells is extremely limited. The most authoritative reference in this area is by Taylor and Camalier (1982). These authors assessed the effect of dissolved oxygen concentration on the growth of epithelial cells from rhesus monkey kidney (cell line LLC-MK) and epithelial cells from human neonatal foreskin. Their results were in contrast to those which have been obtained for fibroblast cells. Epithelial cells were noted to proliferate without inhibition at dissolved oxygen concentrations corresponding to 130 to 140 mm Hg, but were severely inhibited at dissolved oxygen concentrations in the vicinity of 40 mm Hg. The authors hypothesize that a dissolved oxygen concentration of at least 70 mm Hg is necessary for continued epithelial cell proliferation.

No specific OUR values were provided by Taylor and Camalier, but they did demonstrate that the kidney epithelial cells in vitro consumed dissolved oxygen more rapidly than do fibroblast cells.

CARBON DIOXIDE AND EPITHELIAL CELL GROWTH: LITERATURE REVIEW

It has been clearly established that mammalian cells have a carbon dioxide requirement for growth. This has been particularly evident in experimental systems in which pH is controlled via a buffering system other than the carbon dioxide-bicarbonate system. In these cases it has been demonstrated that cells grown in the presence of a carbon dioxide overlay proliferate faster than cells grown in the absence of a carbon dioxide overlay.^{3,5,6,8} The carbon dioxide requirement stems from its incorporation in the anabolic pathways of cells, including the purine and pyrimidine pathways.

In addition to its absolute requirement for molecular synthesis, carbon dioxide has been postulated to serve a major important role as a regulator of cell activity.⁷

The literature would suggest the existence of an optimum external carbon dioxide concentration(s) for cell growth and product formation. In this literature review no data were found concerning this optimum concentration value. It will probably be necessary to determine this value experimentally for each cell line of interest.

The existence of the carbon dioxide requirement does not necessarily mean that a canister of carbon dioxide must be carried on board the Space Shuttle. It is possible that the confluent cells to be carried into space will produce sufficient carbon dioxide to satisfy their own carbon dioxide requirement.

This must be determined experimentally. It is possible that the lag phase associated with the inoculation of cells into the bioreactor could be reduced by the introduction of carbon dioxide from an external during the initial period of cell growth.

MODELLING OXYGEN TRANSPORT AND UTILIZATION

A flow diagram of the NASA bioreactor is presented in figure 6-1. The elements in the main flow loop of the reactor system include the bioreactor, two sensor blocks (containing measurement electrodes for dissolved oxygen, carbon dioxide, and pH), an in-line filter, a mixing chamber, and an oxygenator (designated O_2). A separate system for protein concentration is also indicated in figure 6-1. This loop includes a hollow fiber protein concentration unit and the reservoir for the protein concentrate (designated "protein").

Subscript definitions for each of the volume elements are presented in figure 6-2. Variable assignments for flow rate (F), dissolved oxygen concentration (O), carbon dioxide (C), and volume (V) are presented in figure 6-3. Note that the concentrations of oxygen and carbon dioxide in the liquid stream are specified at the exit of the oxygenator (i.e., O_o and C_o) as an indication that the composition of that stream will change as a function of position in that device.

The oxygenator and the reactor are the only significant volume elements in the main flow loop. At the current time it would appear that these two elements would need to be considered in developing the dynamic oxygen and carbon dioxide mass balances for the system (fig. 6-4). Variable definitions for the inlet and outlet gas streams are included in this figure.

A dynamic model of oxygen transport and utilization is presented in figure 6-5. The assumptions involved in generating these equations will be outlined below.

Equation 5-1

This is a standard dynamic mass balance equation for a continuous stirred-tank reactor (CSTR). It is assumed that the contents of the tank are well-mixed. That is, the liquid contents of the reactor are homogeneous with respect to dissolved oxygen, dissolved carbon dioxide, and cells. The first term of the right side of the equation corresponds to the input and output of oxygen to the reactor via the input and output flow streams. The second term on the right side of the equation corresponds to the oxygen utilization by the cells for growth and maintenance.

Equation 5-2

As a first approximation, the oxygen utilization rate (OUR) is modelled empirically by a simple Michaelis-Menton-type relationship. The choice of this relationship is based on the assumption that the growth of the cells will be dependent on the dissolved oxygen concentration when the concentration is low, and that there will be no inhibition of growth at high levels of dissolved oxygen. The validity of this relationship must be determined through experimentation with the cells of interest. The presence of oxygen inhibition of growth at high concentration levels or the finding of a lower threshold of dissolved oxygen necessary for growth would necessitate the substitution of a more complex relationship for equation 5-2.

The constant K_1 in equation 5-2 corresponds to the maximum possible oxygen utilization rate. As mentioned previously, the experimental values which have been determined (principally for fibroblasts) range from 0.05×10^{-12} to 0.5×10^{-12} moles of oxygen utilized per cell per hour. In the absence of specific experimental data, it would appear to be the most prudent to utilize a value of K_1 of 0.5×10^{-12} moles per cell per hour. This would correspond to a value at the high end of the range which has been observed, in acknowledgment to the findings of Taylor and Camalier (1982) concerning the high oxygen consumption rate of epithelial cells.

The parameter K_2 , the dissolved oxygen concentration at which the OUR is half-maximum, is even more difficult to estimate from the available literature data. In the absence of specific experimental data, a value of 50 mm Hg might be used in simulation--that is $K_2 = 0.0625$, assuming a Henry's Law constant of 0.2 mM/160 mm Hg.

Equations 5-3 and 5-4

In the steady state the oxygen concentrations of the gas and liquid streams of the oxygenator will vary with position in the oxygenator. The dynamic material balances for oxygen on the gas (eq. 5-3) and liquid sides (eq. 5-4) of the oxygenator must therefore be partial differential equations with two independent variables, time (t) and position (z).

These equations describe the situation where the gas and liquid phases pass in "plug flow" through the oxygenator, with negligible back-diffusion or back-mixing (the validity of the plug flow assumption is under examination). The term on the right side of each of these equations represents the transport of oxygen between the gas and liquid phases; as the term indicates, this transport is proportional to the difference at any point between the oxygen concentrations in the gas and liquid streams.

Equations 5-3 and 5-4 reduce to equations 5-3b and 5-4b in the steady state and if it can be assumed that the liquid and gas velocities are constant throughout the oxygenator. Equations 5-3b and 5-4b can be solved analytically to determine the steady state profile of oxygen in the gas and liquid streams, given the entering concentrations of oxygen in gas and liquid streams (see appendix A). Equations 5-3 and 5-4 require a more time-consuming numerical (i.e., computer) solution. Equations 5-3 and 5-4 contain three parameters A_g , A_l , and U_o . The first two parameters, which relate to the area available for mass transport can be computed from the manufacturer's literature. The overall mass transport coefficient for oxygen, U_o , is not available and must be determined through experimentation.

It is possible that the oxygenator will possess such overcapacity for oxygen transfer that the rigorous equations 5-3 and 5-4 can be replaced by a simple equilibrium relationship (eq. 5-5).

A MODEL OF CARBON DIOXIDE PRODUCTION AND TRANSPORT

The dynamic model for carbon dioxide transport in the oxygenator is presented in figure 6-6. Equation 6-1 is the dynamic model describing the dissolved carbon dioxide concentration (C_r) in the reactor. The last term in that equation is the carbon dioxide generation term. The value of the important parameter n , the carbon dioxide production rate (CDPR) per cell, is unknown. It is assumed that the CDPR will be related in some way to the dissolved oxygen concentration--that is, if the dissolved concentration is reduced, then the cell would be expected to produce less carbon dioxide and more lactate. Equation 6-2 represents a first crude approximation of the relationship between the carbon dioxide production rate and the dissolved oxygen concentration.

Equations 6-3 and 6-4 in figure 6-6 represent the dynamic carbon dioxide balances for the liquid and gas streams passing counter-currently through the oxygenator. These equations are analogous in form to equations 6-3 and 6-4 of figure 6-5 for oxygen, and assume negligible back-diffusion or back-mixing of the gas and liquid streams.

Equations 6-3b and 6-4b in figure 6-6 represent the steady state relationships for carbon dioxide on the gas and liquid sides of the oxygenator, assuming that the liquid and gas flow rates in the oxygenator can be assumed to be constant. The same analytical solution technique presented in appendix A for oxygen can be applied to solve these equations for the steady state carbon dioxide profiles in the oxygenator.

For carbon dioxide, no set of conditions is expected to exist which will permit a simplification of the balance equations 6-3, 6-4, 6-3a and 6-4a.

IMPLICATIONS OF THIS INITIAL LITERATURE SURVEY AND MODELLING EFFORT

Based on the model presented previously, it is possible to roughly compare the potential rate of oxygen utilization by cells in the bioreactor with the maximum rate of oxygen transport to those cells in the incoming liquid stream from the oxygenator. Solving equation 5-1 of figure 6-5 in the steady state:

$$0 = F1*(O_o - O_r) - m*X*V_r$$

Assuming values for m , X , V_r , O_o , and O_r of

$$\begin{aligned} m &= 5.0 \times 10^{-13} \text{ moles per cell per hour} \\ X &= 3.0 \times 10^9 \text{ cells per liter} \\ V_r &= 0.5 \text{ liters} \end{aligned}$$

$$\begin{aligned} O_o &= 2.0 \times 10^{-4} \text{ (saturated solution for } pO = 160 \text{ mm Hg)} \\ O_r &= 1.0 \times 10^{-4} \text{ (assumption of desired oxygen concentration at 50\% of} \\ &\quad \text{saturation in the bioreactor)} \end{aligned}$$

These values lead to a required flow rate, F , of

$$F = \frac{(5.0 \times 10^{-13}) * (3.0 \times 10^9) * (.5)}{1.0 \times 10^{-4}} = 7.5 \text{ liters/hour} = 125 \text{ ml/min}$$

The above computation emphasizes the very low capacity of the liquid stream to carry oxygen to the cells in the reactor, due to the low solubility of oxygen in water. A number of factors could result in the necessity of an even higher flow rate:

- If the oxygenator is not capable of saturating the liquid stream
- If it is necessary to have a value of O_r greater than 0.1 mM (e.g., if the optimum dissolved concentration for cell growth or product formation is greater than 0.1 mM.)
- If the solubility of oxygen in cell culture media is less than 0.2 mM for an oxygen partial pressure of 160 mm Hg. This is likely, since the solubility of oxygen in pure water at 37 degrees is 0.2 mM, and the solubility of oxygen is known to decrease with increasing electrolyte concentration. For example, 1.0 M NaCl reduces the solubility of oxygen in water by 30%. The value of 0.2 mM used in the above calculation is therefore close to the maximum possible solubility.
- If the cell concentration is greater than 3.0×10^9 cells per liter

- If the OUR of the kidney cells is greater than 0.5×10^{-12} moles per cell per hour

These factors underscore the importance of reliable experimental data in deciding on a final reactor design. These are several operational and design alternatives which could alleviate any problem which might arise with the current NASA bioreactor configuration:

- Increase the partial pressure of the oxygen in the gas stream to the oxygenator (i.e., by increasing the concentration of oxygen in the feed).
- Redesign the spin filter so that a higher liquid flow rate can be tolerated. The current oxygenator could tolerate much larger flow rates.
- Reduce the vessel size, which would reduce the number of cells which must be supplied with oxygen.
- Design a new oxygenation system which introduces oxygen directly into the reaction vessel (e.g., silicone tubing or sheets in the bioreactor).
- Investigate the possibility of using an artificial oxygen carrier to increase the oxygen-carrying capacity of the liquid stream.

A related issue worthy of consideration is the total gas flow through the oxygenator during long-term operation in space. It is recommended by the manufacturer that the gas flow rate through the oxygenator be roughly two times greater than the liquid flow rate to achieve oxygen saturation. At 50 to 100 ml/minute of liquid flow, the gas flow would be 1000 to 2000 liters per week. If that gas was carried in compressed form at 150 atm, then the gas would occupy about 10 liters. The associated containers and valves would probably raise the total volume to about 20 liters.

The alternative is to use an air pump to circulate cabin air through the oxygenator. This would reduce, but not eliminate, the volume of compressed gas that would have to be carried on the Shuttle. It will be necessary to carry some compressed gas for the following reason. It will probably be desirable to control both the dissolved oxygen and the dissolved carbon dioxide concentrations in the bioreactor. The probable technique for controlling these gases would be to control dissolved carbon dioxide by regulating the total gas flow through the oxygenator (thereby controlling the rate at which carbon dioxide is flushed from the system), and to control the dissolved oxygen concentration by controlling the oxygen concentration in the feed gas stream to the oxygenator. It would therefore be necessary to carry some oxygen and/or nitrogen to achieve the desired oxygen concentration in the gas stream. A final decision concerning which of the two gases would have to be carried on the Shuttle will await experimental results concerning the OUR of the

epithelial kidney cells and the optimum dissolved oxygen concentration for their growth.

It may, additionally, be desirable to carry some carbon dioxide on the Shuttle. Growing cells at low density may not initially produce sufficient carbon dioxide to maintain the carbon dioxide concentration at the desired level. Carbon dioxide would therefore be introduced into the feed gas stream to the oxygenator to avoid an unnecessary lag in cell growth rate. For a confluent culture this may not be a problem.

CONCLUSIONS

A review of the available literature concerning the dissolved oxygen and carbon dioxide requirements of mammalian cells has been presented, and the importance of this data in the design and operation of a mammalian cell bioreactor has been demonstrated. It is noted that little literature data is available for mammalian cells in general, and for epithelial cells in particular. The available literature is dominated by data for fibroblast cell cultures, which appear to be substantially different from epithelial cells in their metabolic requirements. This literature survey underscores the importance of procuring reliable experimental data for the particular cell type of interest.

A preliminary dynamic model has been presented in this report for the simulation of cell growth and oxygen/carbon dioxide transport and utilization. This model will be refined on the basis of experimental data collected from the bioreactor system using the cell type of interest. It is expected that this model will be of utility in the simulation of oxygen and carbon dioxide control algorithms. The model will ultimately be expanded to include product formation by the cells.

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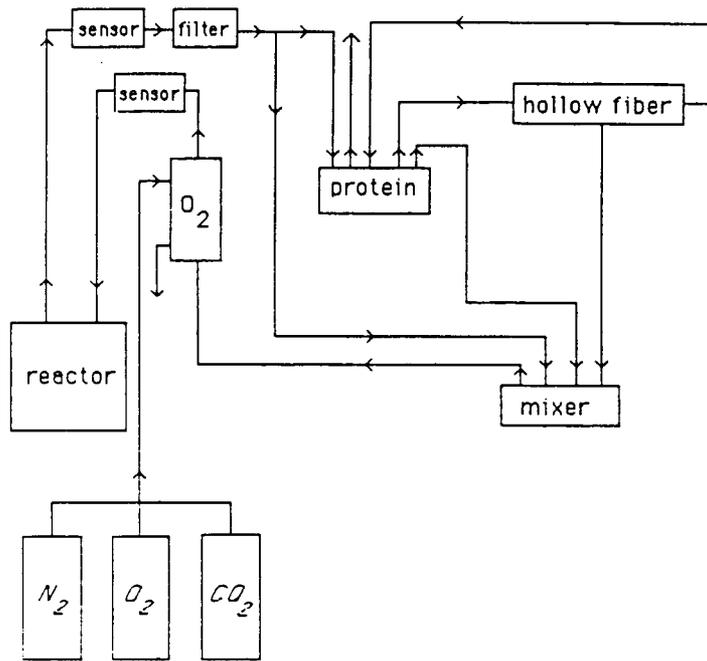


Figure 6-1.- General flow diagram of the NASA bioreactor.

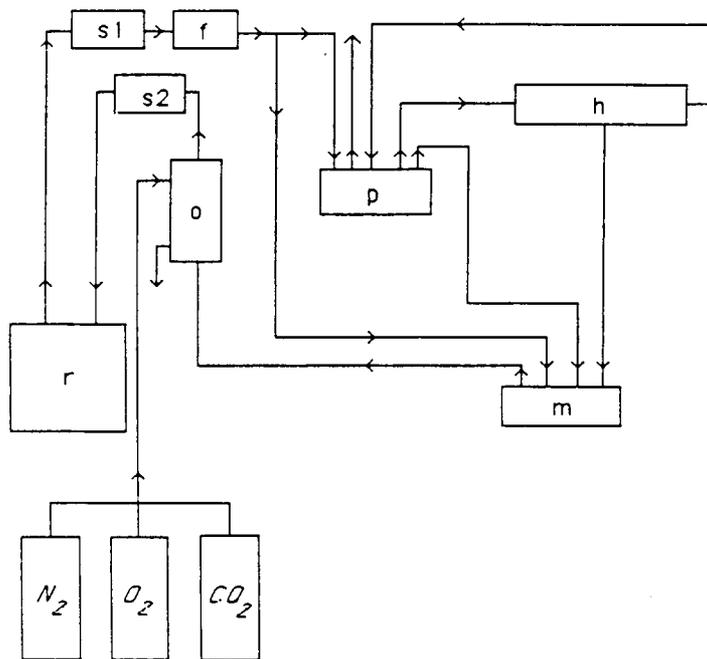


Figure 6-2.- Volume element designations.

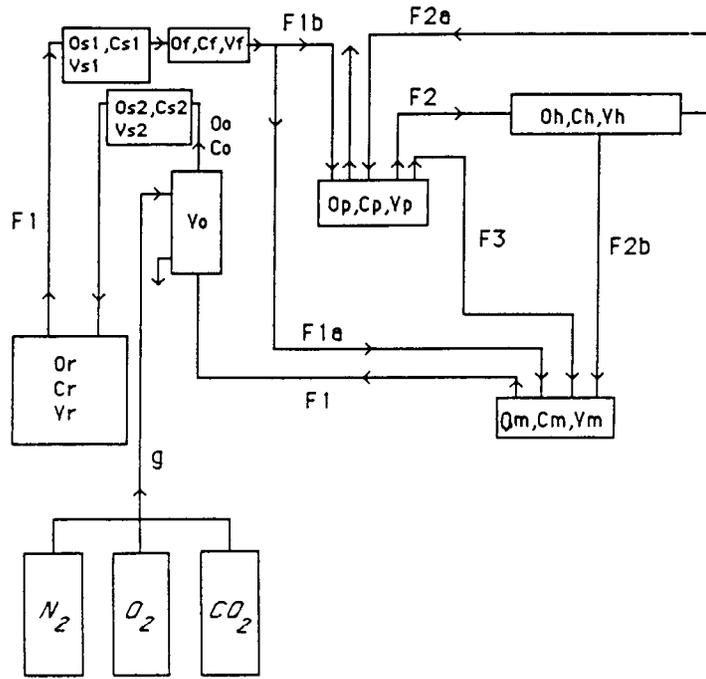


Figure 6-3.- Variable assignments.

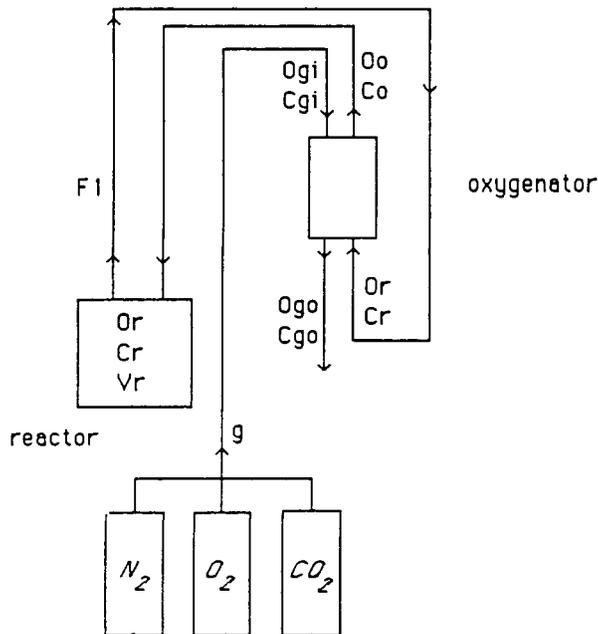


Figure 6-4.- Modelling the dynamics of the main flow loop: dissolved oxygen and carbon dioxide.

$$\text{Reactor:} \quad \frac{V_r dO_r}{dt} = F_1 (O_o - O_r) - m \cdot X \cdot V_r \quad (5-1)$$

$$\text{where} \quad m = \frac{K_1 \cdot O_r}{K_2 + O_r} \quad (5-2)$$

Oxygenator:

$$\text{Gas side} \quad \frac{\partial O_g}{\partial t} + \frac{\partial (V_g \cdot O_g)}{\partial z} = -U_o \cdot A_g \cdot (O_g - O_l) \quad (5-3)$$

$$\text{Liquid side} \quad \frac{\partial O_l}{\partial t} + \frac{\partial (V_l \cdot O_l)}{\partial z} = +U_o \cdot A_l \cdot (O_g - O_l) \quad (5-4)$$

where:

$$O_l = O_r \text{ at } z = 0$$

$$O_l = O_o \text{ at } z = L$$

$$O_g = O_{gi} \text{ at } z = L$$

$$O_g = O_{go} \text{ at } z = 0$$

In the steady state:

$$\text{Gas side} \quad V_g \cdot \frac{dO_g}{dz} = -U_o \cdot A_g \cdot (O_g - O_l) \quad (5-3B)$$

$$\text{Liquid side} \quad V_l \cdot \frac{dO_l}{dz} = +U_o \cdot A_l \cdot (O_g - O_l) \quad (5-4B)$$

$$O_o = \frac{P_o}{K} = \frac{Y_o \cdot P_t}{K} \quad (5-5)$$

where:

V_r = reactor volume

O_r = dissolved oxygen concentration in the bioreactor

O_o = dissolved oxygen concentration leaving the oxygenator

F_1 = flow rate through the bioreactor

m = the oxygen utilization rate (OUR) in units of moles of oxygen per cell per hour

X = cell density in units of cells per liter

K_1 = the maximum oxygen utilization rate

Figure 6-5.- Version 4: Dynamic oxygen balances-main loop components only.

K_2 = the dissolved oxygen concentration at which the OUR is half of maximum
 O_g = the dissolved oxygen concentration in the gas phase at a particular point in the oxygenator
 V_g = the gas phase velocity through the oxygenator
 A_g = the area for gas phase mass transfer per volume of gas in the oxygenator
 U_o = overall mass transfer coefficient for oxygen transport in units of cm/hr
 O_1 = the dissolved oxygen concentration in the liquid phase at a particular point in the oxygenator
 V_1 = the liquid phase velocity through the oxygenator
 A_1 = the area for liquid phase mass transfer per volume of liquid in the oxygenator
 P_o = the partial pressure of oxygen in the gas phase passing through the oxygenator
 Y_o = the mole fraction of oxygen in the gas phase
 P_t = the total pressure of the gas passing through the oxygenator
 K = the Henry's Law constant for oxygen solubility

Figure 6-5.- Version 4: Dynamic oxygen balances-main loop components only (Cont'd).

Reactor:
$$\frac{V_r dC_r}{dt} = F_1 (C_o - C_r) + n \cdot X \cdot V_r \quad (6-1)$$

where
$$n = K_{co2} \cdot O_r(?) \quad (6-2)$$

Oxygenator:

Gas side
$$\frac{\partial C_g}{\partial t} + \frac{\partial (V_g \cdot C_g)}{\partial z} = -U_c \cdot A_g \cdot (C_g - C_l) \quad (6-3)$$

Liquid side
$$\frac{\partial C_l}{\partial t} + \frac{\partial (V_l \cdot C_l)}{\partial z} = +U_c \cdot A_l \cdot (C_g - C_l) \quad (6-4)$$

where:

$$C_l = O_r \text{ at } z = 0$$

$$C_l = O_o \text{ at } z = L$$

$$C_g = C_{gi} \text{ at } z = L$$

$$C_g = C_{go} \text{ at } z = 0$$

In the steady state:

Gas side
$$V_g \cdot \frac{dC_g}{dz} = -U_c \cdot A_g \cdot (C_g - C_l) \quad (6-3B)$$

Liquid side
$$V_l \cdot \frac{dC_l}{dz} = +U_c \cdot A_l \cdot (C_g - C_l) \quad (6-4B)$$

where:

V_r = reactor volume

C_r = dissolved carbon dioxide concentration in the bioreactor

C_o = dissolved carbon dioxide concentration leaving the oxygenator

F_1 = flow rate through the bioreactor

n = the carbon dioxide production rate in units of moles of carbon dioxide per cell per hour

K_{co2} = a proportionally constant for the hypothetical relationship of equation 6-2 above

X = cell density in units of cells per liter

C_g = the dissolved oxygen concentration in the gas phase at a particular point in the oxygenator

Figure 6-6.- Version 4: Dynamic carbon dioxide balances-main loop components only.

- Vg = the gas phase velocity through the oxygenator
- Ag = the area for gas phase mass transfer per volume of gas in the oxygenator
- Uc = overall mass transfer coefficient for carbon dioxide transport in units of cm/hr
- Cl = the dissolved oxygen concentration in the liquid phase at a particular point in the oxygenator
- VI = the liquid phase velocity through the oxygenator
- Al = the area for liquid phase mass transfer per volume of liquid in the oxygenator

Figure 6-6.- Version 4: Dynamic carbon dioxide balances-
main loop components only (Cont'd).

FED-BATCH CONTROL BASED UPON THE MEASUREMENT
OF INTRACELLULAR NADH

W. B. Armiger, J. F. Lee, L. M. Montalvo
and J. R. Forro
BioChem Technology, Inc.
Malvern, PA

INTRODUCTION

This paper describes a series of experiments demonstrating that on-line measurements of intracellular NADH by culture fluorescence can be used to monitor and control fermentation processes. A distinct advantage of intracellular NADH measurements over other monitoring techniques such as pH and dissolved oxygen is that it directly measures real-time events occurring within the cell rather than changes in the environment and when coupled with other measurement parameters can provide a finer degree of sophistication in process control.

Culture fluorescence measurement techniques were first introduced by Duysens and Ames.¹ They noted that the fluorescence spectrum for suspensions of aerobic baker's yeast was similar to the one for NADH and that the fluorescence signal could be significantly enhanced by additions of ethanol or glucose. Damped oscillations in fluorescence were observed after the establishment of anaerobic conditions. These oscillations were similar to those reported earlier by Chance² in yeast using absorption spectrophotometry. Chance and Thorell³ found that most cellular fluorescence in eucaryotic cells was localized within mitochondria where NADH is concentrated because of its role in respiration. Chance et. al.⁴ investigated the initiation of fluorescence oscillations in suspensions of starved yeast. The oscillations are caused by the sudden rates of glycolysis (Pasteur Effect). This work established a laboratory technique to follow intracellular metabolism.

Harrison and Chance⁵ later built an instrument capable of measuring culture fluorescence within a fermentor. With this system, they were able to monitor aerobic/anaerobic transitions in continuous cultures of Klebsiella aerogenes. Using a similar device, Zabriskie and Humphrey⁶ established a linear relationship between the log of culture fluorescence and the log of cell concentration. Ristroph et. al.⁷ studied the relationship between culture fluorescence and the growth of Candida utilis in a fed-batch fermentation during which ethanol was added in discrete pulses. In this process, excessive ethanol feeding rates cause acetate to accumulate as a by-product. In the absence of ethanol, the yeast can assimilate the accumulated acetate. After each ethanol pulse, culture fluorescence peaks and declines gradually

as the ethanol is consumed. At the point where ethanol is exhausted and acetate consumption begins, the fluorescence declines more rapidly. Corresponding changes in the respiratory quotient indicate the metabolic changes accompanying the diphasic growth. The reproducible response of culture fluorescence to ethanol and acetate metabolism suggests that the instrument could be used to control the ethanol feeding schedule to improve process yield and productivity.

NADH IN METABOLISM

A highly simplified summary of basic cellular metabolism, illustrated in figure 7-1, shows how NAD⁺ is constantly cycled between the oxidized form (NAD⁺) and the reduced form (NADH). As the organism utilizes substrate and converts it into carbon dioxide, metabolic intermediates and other catabolites, NAD⁺ is reduced to NADH. In aerobic cells, oxygen is used to reoxidize the NADH to NAD⁺ during oxidative phosphorylation and energy in the form of ATP is produced for use in other metabolic reactions (e.g., growth). Anaerobic organisms operate in a similar manner, but use electron acceptors other than oxygen.

Figure 7-1 may also be used to illustrate the effect of various parameters on the reducing level of the cell - that is, the ratio of NADH concentration to the total NADH + NAD⁺ concentration in the cell. If in an aerobic system the oxygen supply is limited while there is an adequate supply of substrate present, catabolism will continue to function, but reoxidation of NADH by respiration stops. Under these conditions the reducing level approaches unity. If on the other hand, substrate becomes limiting or depleted, NADH levels fall and the reducing level approaches zero.

By measurement and control of the reducing state of the cell (i.e., the ratio of NADH to total NADH + NAD⁺), it is possible to maintain the culture in the desired condition for selected growth or metabolic activity.

THE FLUORESCENT SIGNAL

The fluorescent signal received from a fermentation culture is a composite signal which is a function of the number of cells, the reducing state of the cell, and environmental effects. This is illustrated in figure 7-2 for a fed-batch process which may be viewed as a high frequency signal superimposed on a low frequency signal. If the signal is properly decomposed it can reveal information regarding the process. The cell concentration and environmental effects will vary slowly while the metabolic effects (reducing state) due to varying nutrient or oxygen can change rapidly. Decomposition

of the fluorescent signal into its major components can then yield data for process control.

FERMENTOR STUDIES

Figure 7-3 is data obtained during growth of a mammalian cell culture showing fluorescence (NFU values), total cell count (-), percent viability (+), and temperature. Figure 7-4 depicts similar results obtained with a Pediococcus and shows NFU, pH, and viable cell counts (\square). In both cases there is a good correlation between cell growth and measured culture fluorescence, but it is also evident that the fluorescent signal has other components - lack of correlation between cell count and fluorescence in early stages of Pediococcus growth was due, for example, to pH effects on cells and background (environmental).

Actual data illustrating how the signal can be decomposed into its specific elements is shown in the next series of figures. Figure 7-5 shows the response of a culture of the yeast Saccharomyces to changes in dissolved oxygen levels. Yeast cells were placed in a nongrowth environment and allowed to metabolize their internal glycogen reserve - yielding a relatively constant fluorescent signal.

At 1.5 hours the air to the fermentor was turned off. As the DO level began to fall and approach zero, there was a step increase in the culture fluorescence signal due to an increase in intracellular NADH. This corresponds to the organism undergoing a metabolic transition from an aerobic to an anaerobic state. It should be noted that the step increase in fluorescence actually occurs prior to a DO reading of zero. This is a consequence of the more rapid response of the FluroMeasure™ Detector relative to a DO probe. By the FluroMeasure™ monitoring intracellular conditions, it indicates that the cell is internally anaerobic before the broth is depleted of oxygen. The air flow was turned back on at 2.0 hours and there was a rapid decrease in the NFU reading back to the level corresponding to aerobic metabolism. After 2.0 hours the culture continued to be in a resting condition. After 2.5 hours there was a rapid decrease in the fluorescence (NFU) signal as the glycogen stores of the yeast became depleted. At approximately 3.0 hours the DO returned to 100% and the level of culture fluorescence (NFU) reached its minimum energy value as metabolism ceased.

In the absence of glucose and glycogen, yeast will aerobically metabolize ethanol via the pathway illustrated in figure 7-6. In this pathway ethanol is metabolized through acetaldehyde to acetate and in both reactions NADH is produced. Acetate is further metabolized through acetyl-CoA into the TCA cycle.

Using high pressure liquid chromatography to monitor the change in extracellular ethanol and acetate concentrations, an ethanol addition cycle was studied and the results are shown in figure 7-7. At 2.4 hours ethanol was added at a concentration of 250 PPM. The ethanol was rapidly absorbed by the cells and its concentration dropped to less than 100 PPM within the first minute after its addition. This was followed by a more gradual decline in the ethanol concentration between 2.5 and 3.0 hours until ethanol was entirely depleted. It should be noted that when the extracellular ethanol concentration is greater than 50 PPM, there is a steady state condition in the NFU signal. As the ethanol concentration drops below this level and approaches the K_s value, it becomes rate limiting and there is a rapid decrease in the level of intracellular NADH. This indicates that the cells are in a transition from one steady state reducing level to another lower reducing level. At 3 hours there is an inflection point corresponding to the exhaustion of ethanol. During this same time period there is an accumulation of acetate in the media. The acetate concentration, which was initially zero, increases to greater than 100 PPM while the ethanol is being utilized. When the ethanol is depleted, acetate re-enters the cell and is then metabolized. Depletion of the acetate corresponds to the next inflection point in the curve at 3.15 hours.

Extending this data to various cell concentrations it is found that a series of curves can be developed relating the NFU value to the active catabolic pathways. This is shown in figure 7-8. The bottom curve, representing the lowest reducing state for the culture, is the NFU values for cells which are in the resting state devoid of metabolizable substrate. The top curve, representing the highest steady state cellular reducing level, is derived from the NFU value for cells converting glucose to ethanol under anaerobic conditions. Similar curves have been generated for aerobic conversion of ethanol to acetate and for acetate metabolism. For any cell concentration, the on-line NFU value can be used to determine the active metabolic pathways. Thus, the reducing state of the cell is a basis for process control.

Fed-batch fermentations with Saccharomyces were conducted using a glucose feed rate based on a known starting biomass concentration and an estimated biomass doubling time. Figure 7-9 shows the NFU and cell mass data for one of these runs. For the first 6 hours after inoculation NFU levels remained constant and then began to rise. Cell mass increased only slightly during this time. The sharp drop off in the NFU value at about 7.5 hours was due to stoppage of the glucose feed. The glucose feed was restored at about 9 hours and both cell mass and NFU readings continued to increase.

For each cell mass value measured in this experiment a corresponding minimum (resting cell) and maximum (glucose to ethanol pathway) reducing value was determined by reference to figure 7-8. Reducing levels for aerobic ethanol utilization were also determined (+-). These values were then plotted along

with the NFU readings from the fed-batch fermentation (fig. 7-9) and are shown in figure 7-10. During production of yeast, 50% of the available glucose theoretically can be converted to cell mass. Production of ethanol results in reduced yields and lower productivity. For optimal yeast cell mass production, therefore, the reducing state should be controlled between the two extreme conditions.

As can be seen from figure 7-10 initial NFU values were high, indicating overfeeding with resulting ethanol production. This was confirmed by off-line HPLC analysis for ethanol. As cell mass began to increase, the NFU reading remained constant and approached a midpoint range between the two extreme values. At about 7.5 hours, stoppage of the feed resulted in an immediate drop in NFU values to the resting cell level. When the feed was restarted at about 9 hours, NFU values increased immediately and then dropped again. From about 11 to 12 hours into the run the NFU values indicated a proper balance between substrate consumption and increase in cell mass. Beginning at about 12 hours; however, an imbalance between these two parameters occurred again resulting in the production of ethanol as evidenced by the increase in NFU values and confirmed by HPLC analysis. A series of oscillations was then noted in which additional ethanol was produced indicative of the Crabtree effect.

From about 15 to 20 hours into the run the glucose feed rate was constant and the cell mass increased to a level where it was no longer being overfed. At this stage the glucose feed was stopped and NFU values dropped and remained at a level corresponding to aerobic ethanol metabolism.

CONCLUSION

These data demonstrate that the measurement of intracellular NADH can be utilized to monitor the internal reducing state of cells. The internal reducing state reflects the major metabolic events which are occurring in the culture. Since these data describe pathways which are common to those found in many procaryotic and eucaryotic organisms, similar information may be used to control a wide variety of fermentations and cell cultures.

It should be possible to develop control algorithms for product induction or feeding strategies to maintain a culture at a selected reducing level corresponding to the desired product output.

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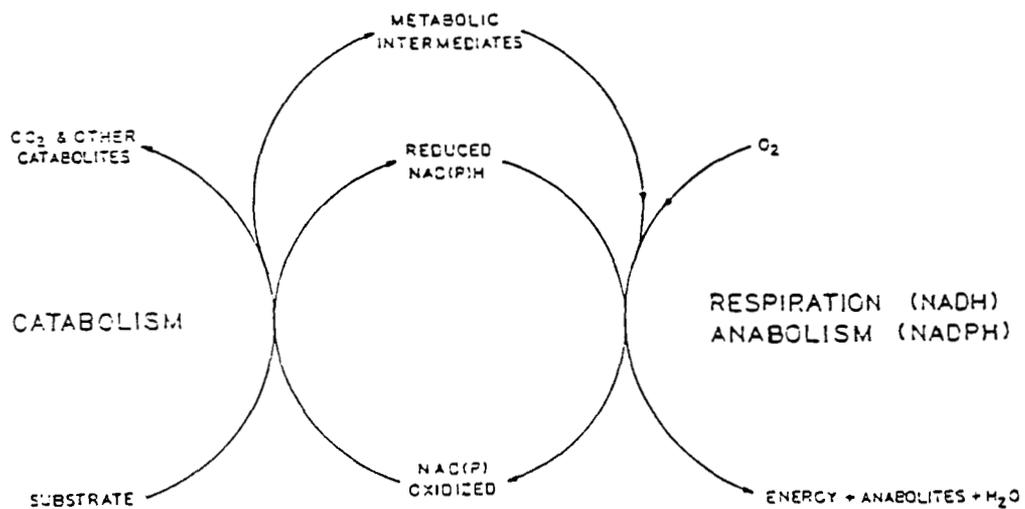


Figure 7-1.- Diagram of basic cellular metabolism showing involvement of oxidized and reduced pyridine nucleotides.

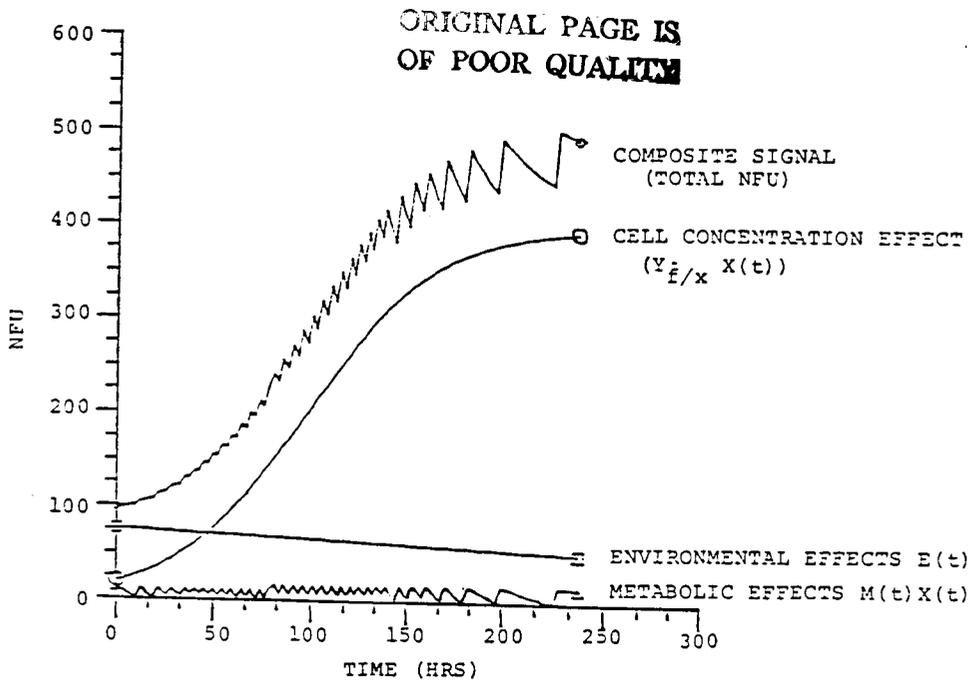


Figure 7-2.- Representation of culture fluorescence as a function of the number of cells, the reducing state of cells, and environmental effects.

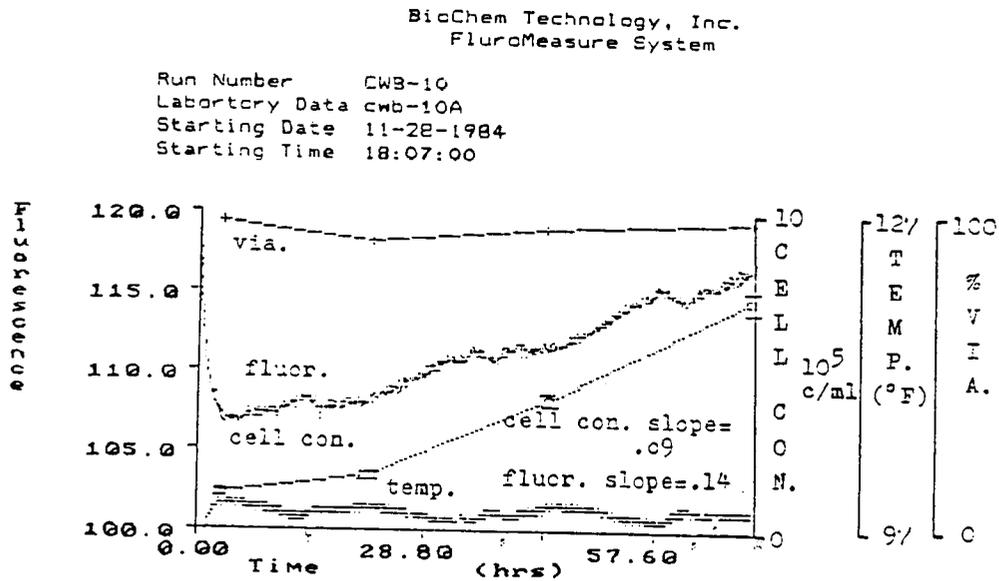
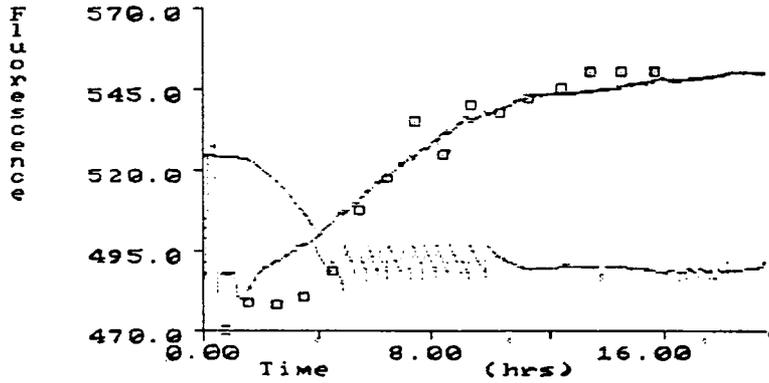


Figure 7-3.- Fluorescent changes during growth of Mouse L29 cells in suspension culture.

BioChem Technology, Inc.
FluoroMeasure System

Run Number MLT-07A
Laboratory Data MLT-07AL
Starting Date 06-26-1985
Starting Time 07:40:00



The Range of Variables in MLT-07A

Variable Name	Range	Unit
Fluorescence	470.00--- 570.00	NFLU
p H	5.50--- 7.50	

The Range of Variables in MLT-07AL

Variable Name	Range	Unit
□ cell con	0.00--- 200.00	c/ml*10 ⁷

Figure 7-4.- Fluorescent changes during growth of a Pediococcus sp.

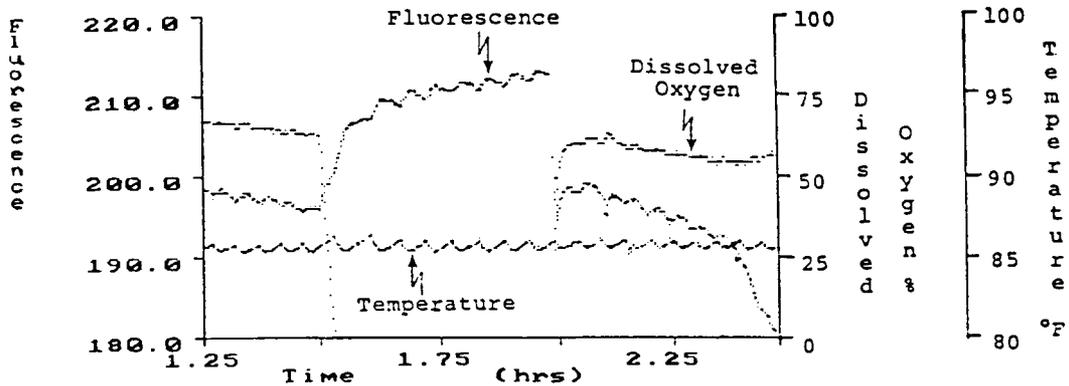


Figure 7-5.- Fluorescent response of Saccharomyces to changes in dissolved oxygen levels.

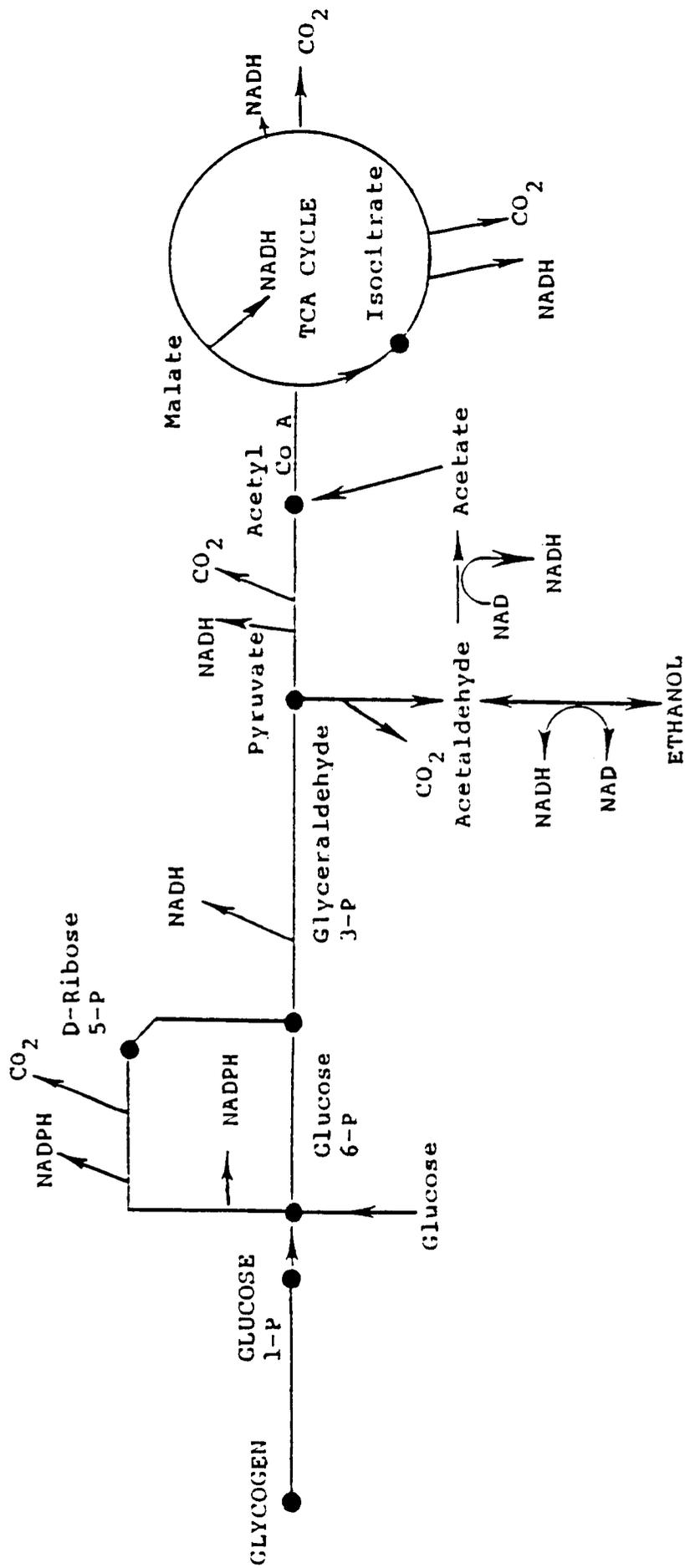


Figure 7-6.- Aerobic ethanol metabolism.

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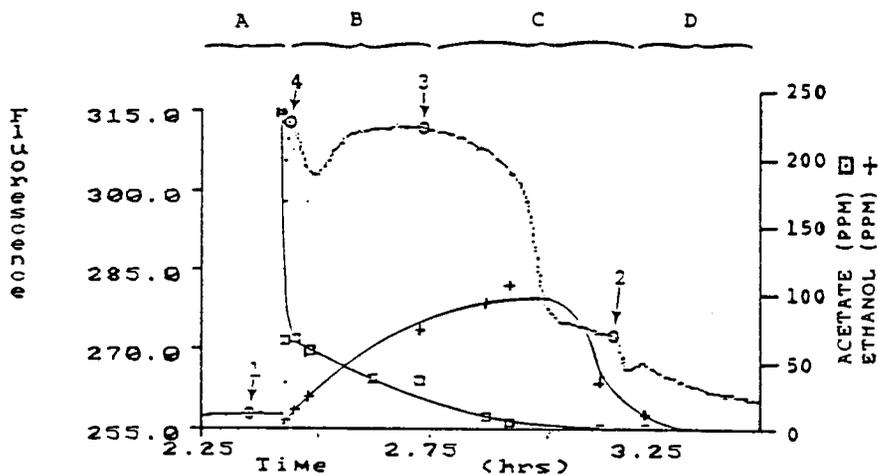
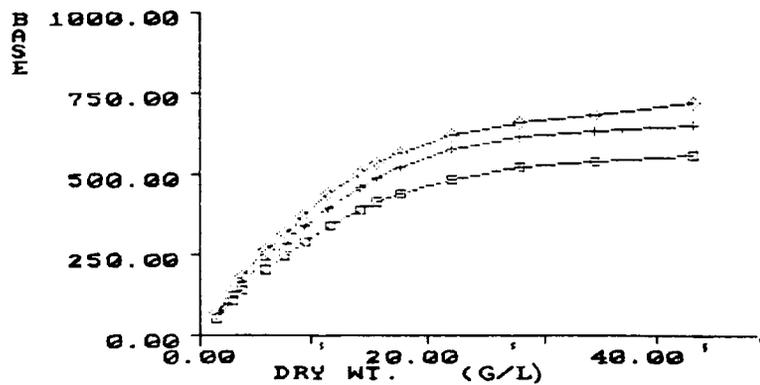


Figure 7-7.- Correlation of fluorescence with ethanol and acetate concentration during ethanol metabolism in starved Saccharomyces.

Run Number
Laboratory Data REGRESS
Starting Date
Starting Time

BioChem Technology, Inc.
FluoroMeasure System



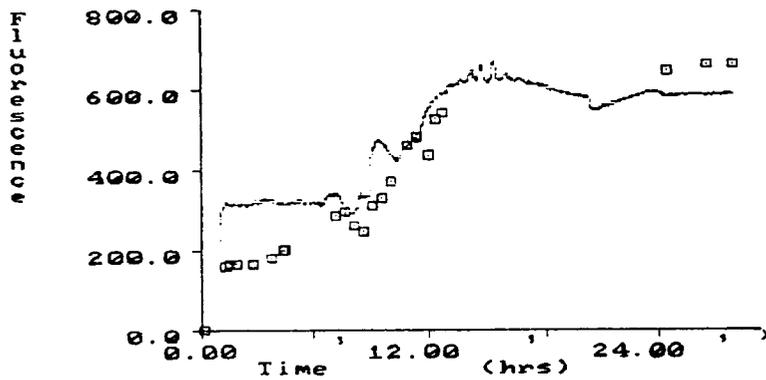
The Range of Variables in REGRESS

Variable Name	Range	Unit
□ BASE	0.00---1000.00	NFU
+ ETOH	0.00---1000.00	NFU
◇ ANAEROBE	0.00---1000.00	NFU

Figure 7-8.- Fluorescence values at various cellular reducing states as a function of dry cell mass.

Run Number
 Laboratory Data mf-06a1
 Starting Date
 Starting Time

BioChem Technology, Inc.
 FluoroMeasure System



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The Range of Variables in

Variable Name	Range	Unit
Fluorescence	0.00--- 800.00	NFU

The Range of Variables in mf-06a1

Variable Name	Range	Unit
■ DRY WT.	0.00--- 40.00	G/L

Figure 7-9.- Fluorescence and cell mass changes during fed-batch growth of Saccharomyces on glucose.

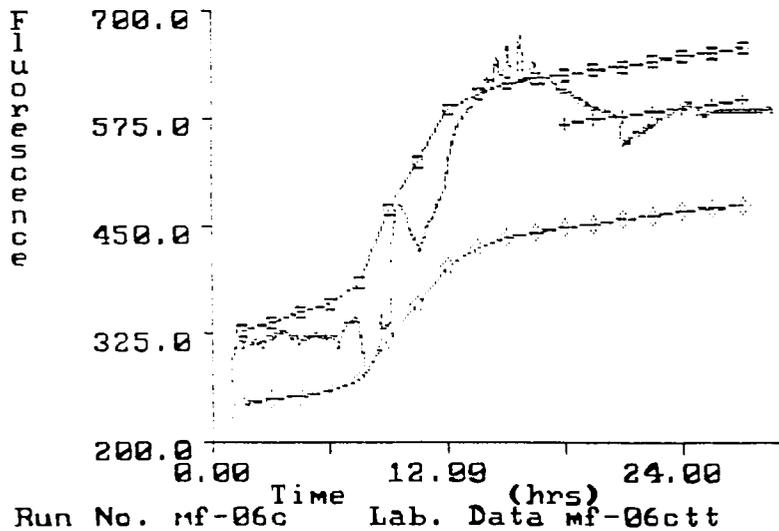


Figure 7-10.- Measured fluorescent values versus theoretical minimum and maximum values for a Saccharomyces fed-batch glucose fermentation.

NUTRIENT REQUIREMENTS AND OTHER FACTORS INVOLVED IN THE CULTURE OF HUMAN
KIDNEY CELLS ON MICROCARRIER BEADS

Marian L. Lewis, Ph.D.
Technology Inc.
Houston, Texas

Dennis R. Morrison, Ph.D.
NASA - Johnson Space Center/SD4
Houston, Texas

This report describes the culture of human kidney cells on microcarrier beads in the Bioprocessing Laboratory at NASA/Johnson Space Center. These were the first series of studies done before and during 1983 to determine optimum culture conditions including medium type and bead type and density. The composition of several medium types and the molecular weights of some common culture medium supplements and cellular proteins are included for information. The report also describes the microgravity cell-to-bead attachment experiment performed on STS-8.

CELLS AND BEADS

The human kidney (HK) cells used in these studies were obtained from a commercial vendor (M. A. Bioproducts) as monodisperse frozen primary cells. The cultured cells were characterized in our laboratory as normal human diploid, anchorage dependent cells with a finite lifespan of 8.3 population doublings. This cell type was chosen because of production of high levels of urokinase (UK), heterogeneity of electrophoretically separable subpopulations and relative ease of culture. Four morphological subtypes were identified microscopically as fibroblastoid, fenestrated, and large and small epithelioid cells. The diameter size range is approximately 10 to 30 microns and the generation time in culture is 24 hours.

The microcarrier beads, Cytodex 3, used in these studies were obtained from Pharmacia Fine Chemicals and consisted of a thin layer of denatured collagen chemically bonded to a cross-linked dextran matrix.² Table 8-1 shows characteristics of these microcarriers.

SELECTION OF CULTURE MEDIUM

Several types of commercially available media were tested for their ability to support cell growth in our laboratory. These media included M-199, Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium Alpha (MEM- α), and a medium developed in our laboratory, Microcarrier Medium (MM), which

consisted of the above listed media plus other additives. Components of Microcarrier Medium are shown in table 8-2. Before mixing with cells, the media were supplemented with 10% fetal calf serum, 100 units and 100ug per ml of penicillin and streptomycin.

Figure 8-1 shows results of a study comparing media to determine which type produced the best outgrowth. The cells were seeded at low density of about 50,000/ml. Counts showed a two-day attachment and lag period followed by log growth to day 7. In this test the medium was not changed to replace depleted nutrients and the cells were degenerating by day 10 before reaching a high density. Based on this and repeated tests showing the same result, we selected MM as the medium of choice for cell growth. Although DMEM did not support outgrowth as well as MM, the cells appeared to survive longer. This medium would be a candidate in cases which slower growing cells would be desirable.

COMPARISON OF MICROCARRIER TYPES

A series of studies were done to define cell growth on several different types of microcarriers. Figure 8-2 shows results of one such study. Biosilon beads (Nunc) are polystyrene, Cytospheres (Lab-Tek Division, Miles Laboratories) are non-toxic plastic surface treated beads, and Cytodex 1, 2, and 3 are cross-linked dextran matrix (Pharmacia Fine Chemicals). Cytodex 1 beads bear charged DEAE groups throughout the bead and Cytodex 2 beads have a surface layer of charged DEAE. The Cytodex 3 beads have a collagen layer bonded to the surface of the beads.

Figure 8-2 shows that cells seeded in plates with the Cytodex 3 beads attached and grew much better than those seeded on Cytodex 1, 2, or Cytospheres. Biosilon beads appeared to be a second choice if it should become desirable to culture cells on polystyrene beads. No follow-up work has been done to define cell survival for longer than 7 days on the Biosilon beads.

EFFECT OF BEAD CONCENTRATION ON CELL SURVIVAL

To determine the survival of cells in a no-headspace system at different concentrations, cultures were set up using passage 3 HK cells. When the cells were about 90% confluent on the beads, the beads were divided into suspension sets containing 10, 20, and 30 mg/ml. These suspensions were then transferred to syringes and placed in the incubator. As shown in figure 8-3, the survival of cells in syringes with beads at 10 mg/ml was superior to the higher concentrations. This was expected since oxygen depletion would occur more rapidly at higher cell concentrations. The time at which approximately half of the cells were no longer on the beads for each concentration was two

days for beads at 30 mg/ml, 2.75 days for beads at 20 mg/ml, and 4 days for beads at 10 mg/ml. These studies were preliminary to the more sophisticated experiments designed later to define the oxygen usage of the HK cells.

NORMAL CULTURE CONDITIONS DEVELOPED FROM THESE STUDIES

Based on these studies, the cells are normally grown on the Cytodex 3 beads in static petri plate cultures. The initial seeding density for beads is 5 mg/ml and cells are set up at 50,000/ml. The total volume per dish is 20 ml. Cells are usually confluent in 7-10 days and the cell yield is about 300,000 to 500,000 cells/ml depending on passage level and general condition of the cells when they are set up.

ATTACHMENT OF CELLS TO BEADS IN MICROGRAVITY

Having defined the optimal parameters of medium type and bead type and density for growth of the KH cells in our laboratory, we wanted to determine the behavior of the cells in microgravity. In 1983, we developed an experiment to determine if cells will attach to beads in space. Since these anchorage dependent cells will not survive in a suspended state, it is important to determine if they will attach to substrata in the microgravity environment. Potential applications of this information include providing growth surfaces to cells separated by continuous flow electrophoresis and directing seeding cultures in the bioreactor while on-orbit.

On STS-8, cells and beads were mixed on-orbit in incubated growth chambers.^{3,4} At selected time intervals, glutaraldehyde was injected into the chambers and after the flight, cell attachment was evaluated. Counts of beads with cells attached showed that the cells attached to beads as well and possibly better in microgravity than in the ground-based control experiment. Figure 8-4 shows a scanning electron micrograph of a cell attached to a bead surface from a ground control sample fixed 5 minutes after mixing cells and beads. After 24.5 hours post mixing the beads with cells, some bead surfaces were almost confluent in both the flight and ground experiment as shown in figure 8-5. There were no apparent differences in the manner in which the cells were attached between the flight and ground control. This preliminary experiment showing that it is possible to seed cultures in space is a precursor for future microgravity bioreactor experiments.

CULTURE MEDIUM CONSIDERATIONS FOR THE BIOREACTOR

Nutrient depletion is a primary consideration in the mass culture of cells. In order to replenish those components which are rapidly utilized by the

cells, it is desirable to define the amounts in culture medium before and during cell culture. This may be done for the amino acids by HPLC. Figure 8-6 shows a typical amino acid profile. The figure translates into the actual concentrations shown in table 8-3 for a cell culture maintained on serum free medium for 16 days. As the bioreactor project develops, experiments will be done to define a plan for resupplying depleted amino acids and other nutrients. In cases in which it is desirable to grow the cells in the bioreactor instead of just maintaining them, additional growth supplements such as fetal calf serum must be added to the medium. With addition of serum, the medium is no longer defined, thus evaluating nutrient depletion becomes complicated. One way to reduce the ambiguities due to serum is to use defined serum such as that obtainable from HyClone.

In addition to the components listed for the HyClone serum there are other supplements which are sometimes found in cell culture media. Some of these are listed with their molecular weights in table 8-4. Table 8-5 shows the molecular weights of some proteins found in cells. The high molecular weight proteins must be considered in the product concentration loop design since several are in the same range as the plasminogen activators and other natural cell products which have been suggested for concentration and harvest.

CONCLUSIONS

Human kidney cells have been successfully cultured on microcarrier beads in a growth medium developed in our laboratory. These studies were done using light seeding densities of approximately 2-3 cells per bead and the cells were grown in static petri plate cultures. Seeding cultures at 50,000 cells/ml and a bead density of 5 mg/ml resulted in a lag period of about 2 days before beginning of the log growth phase. Cultures reached confluence in 7 to 10 days.

Having defined the optimum culture conditions in ground based studies, cell attachment experiments were flown on STS-8 which showed that cell attachment in microgravity equaled and was perhaps superior to that on Earth. These studies are preliminary to development of the cell biology experiments being designed for the flight bioreactor.

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TABLE 8-1.- CHARACTERISTICS OF MICROCARRIERS

<u>Type</u>	<u>Cytodex 3 (Pharmacia)</u>
Density (in 0.9% NaCl)	1.04 G/ML
Size (mean diameter)	175 microns
Size (range)	133-215 microns
Approximate area	4,600 cm ² /g dry weight
Approximate number beads/g dry weight	4.0 × 10 ⁶
Swelling factor (in 0.9% NaCl)	14 ml/g dry weight

Cytodex 3 beads consist of a thin layer of denatured collagen chemically coupled to a matrix of cross-linked dextran.

TABLE 8-2.- MICROCARRIER MEDIUM

<u>Component</u>	<u>MFR</u>	<u>Weight or Volume</u>
M-199	Gibco	1 pkt
Mem-Alpha	Gibco	1 pkt
DME (w/4.5 g glucose)	Gibco	1 pkt
NaHCO ₃	Sigma	16.2 g
Bactopectone	Difco	1.2 g
Folic Acid	Sigma	0.02 g
i-Inositol	Sigma	0.072 g
Nicotinic Acid 0.5 mg/ml	Sigma	2 ml
Deionized High Purity H ₂ O	-	2776 ml
Fetal Bovine Serum	-	300 ml
Penicillin/Streptomycin	-	100 units/100ug/ml Final concentration
Hepes	-	10 mM Final concentration

TABLE 8-3.- AN EXAMPLE OF AMINO ACIDS IN SERUM-FREE MEDIUM
CONDITIONED BY HEK CELLS

Sample: T25616
I.D. Run Number: 18-181
Date: July 1, 1985

<u>Amino Acid</u>	<u>Amount Applied</u> <u>(Nanomole)</u>	<u>Concentration</u> <u>(Micromole/ml)</u>
Carboxymethyl-cys	1.229	0.54
ASP	1.095	0.48
THR	2.842	1.25
GLN	*	0.00
SER	2.339	1.03
GLU	3.366	1.48
PRO	1.306	0.57
GLY	289.13	127.22
ALA	1.371	0.60
CYS	0	0.00
VAL	2.756	1.21
MET	0	0.00
ILE	2.158	0.95
LEU	4.971	2.19
TYR	.962	0.42
PHE	1.54	0.68
HIS	.507	0.22
LYS	3.924	1.73
ARG	1.176	0.52

*GLN is not resolved. It co-elutes with THR.

TABLE 8-4.- MOLECULAR WEIGHTS
Some Supplements to Cell Culture Medium

<u>Component</u>	<u>MW</u>
Human fibronectin	~220,000
Transferrin	~85,000
Insulin (bovine)	5,700
Selenous acid ($\text{Na}_2\text{S}_\text{E}\text{O}_3$ or NA selenite)	
Ascorbic acid	176.1
Glutamine	146.1
Glucose	~180
BSA	70,000
Ethanolamine	~142
Lipids (Phospho-)	750
Alanine	89
Lactic acid	~101
Ficol 400	400,000
Epidermal growth factor	6,100
Dextran T-70	~70,000
Dextran T-500	~500,000
Fibronectin	~220,000
Poly-D-Lysine	~70,000

TABLE 8-5.- MW'S OF SOME CELLULAR PROTEINS (5,000 1×10^6 RANGE)

Some Cellular Enzymes

Tryptophan synthetase		159,000
Aspartate transcarbamylase (<u>E. Coli</u>)		310,000
Glutamine synthetase (<u>E. Coli</u>)		592,000
Pyruvate dehydrogenase complex		7×10^6
Polypeptides	100 - 300 AA's	12,000-36,000

Some Serum Components

Serum albumin	550 AA's	68,000
α_1 -Globulins		40,000-55,000
α_1 -Lipoproteins		200,000-400,000
α_2 -Globulins		Up to 800,000
β_1 -Lipoproteins		$3-20 \times 10^6$
γ Globulins		150,000
Fibrinogen		340,000
Plasminogen		90,000

Other

Myoglobin (prob. not in our cells)		16,900
Myosin		470,000

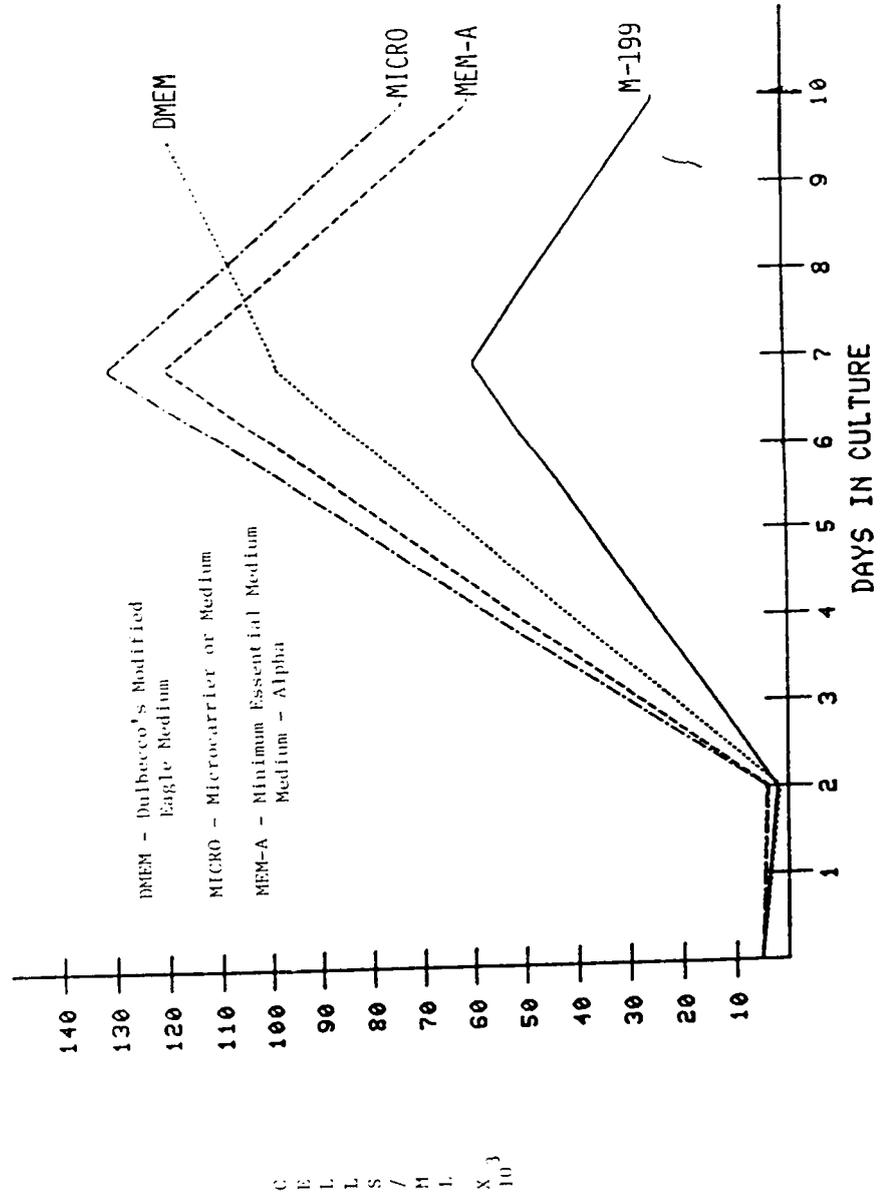


Figure 8-1.- Comparison of medium types for outgrowth of cells on Cytodex 3 microcarriers.

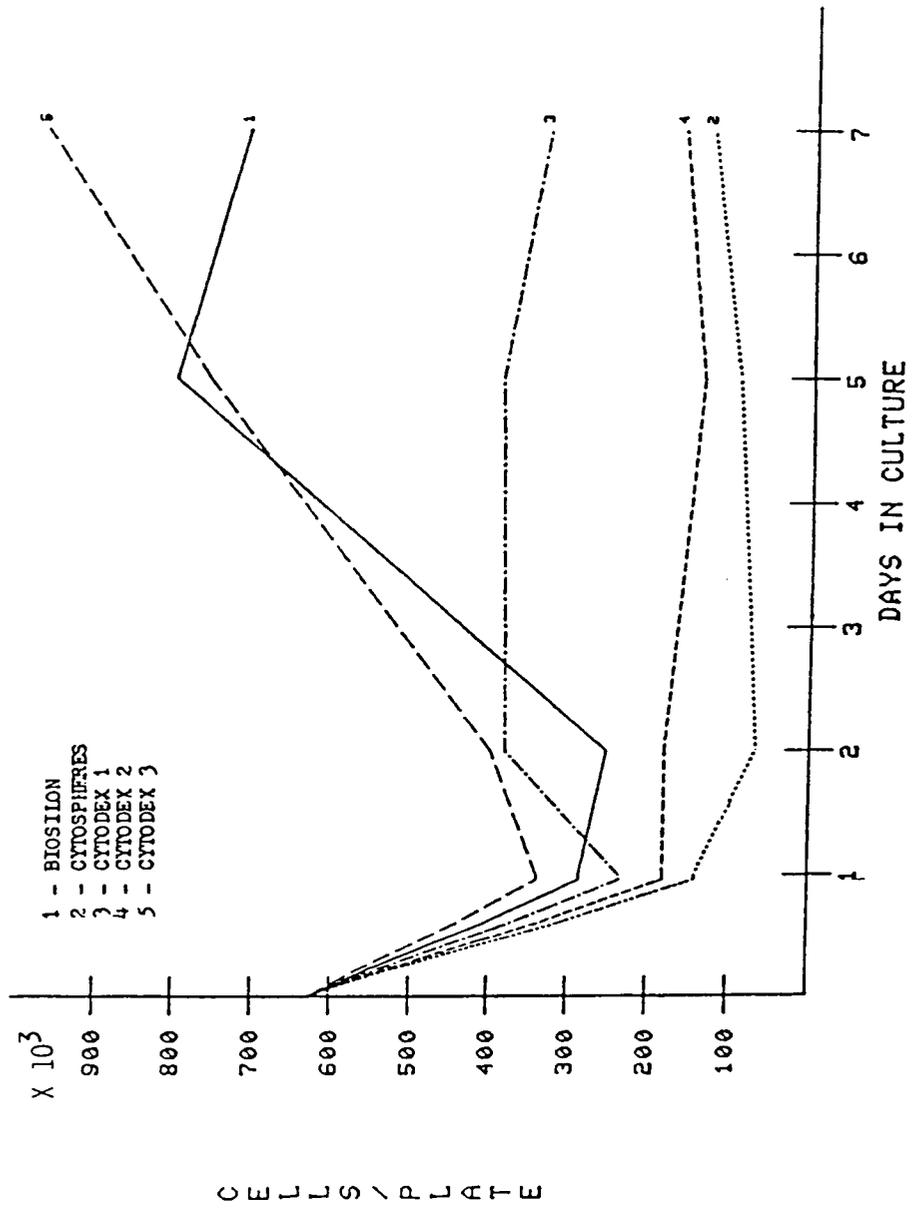


Figure 8-2.- Microcarrier comparison study.

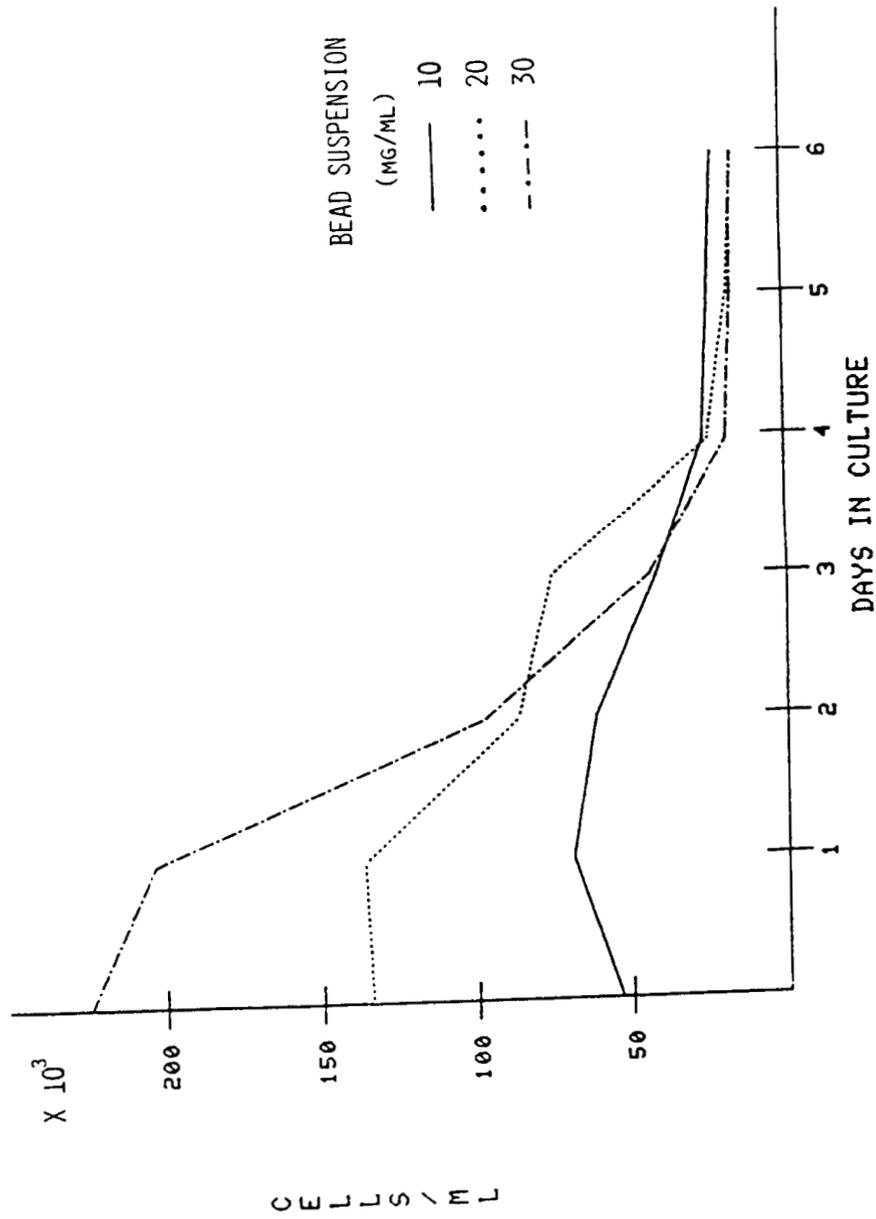


Figure 8-3.- Microcarrier syringe cultures HEK 4347 P-3 on Cytodex 3-90% confluent.

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Figure 8-4.- Ground control-fixed 5 min. after mixing cells and beads.

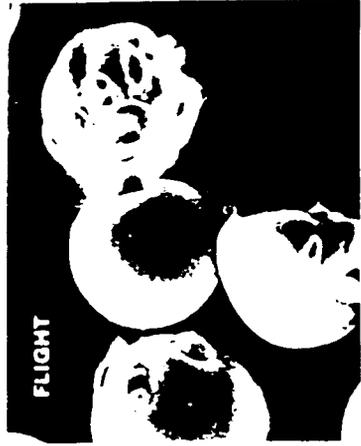
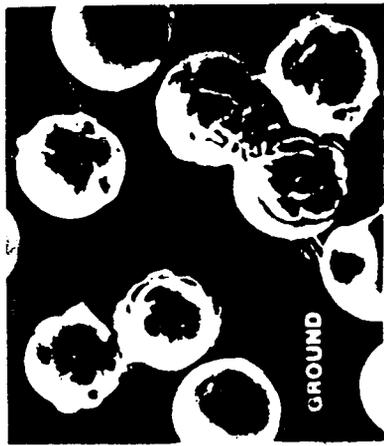
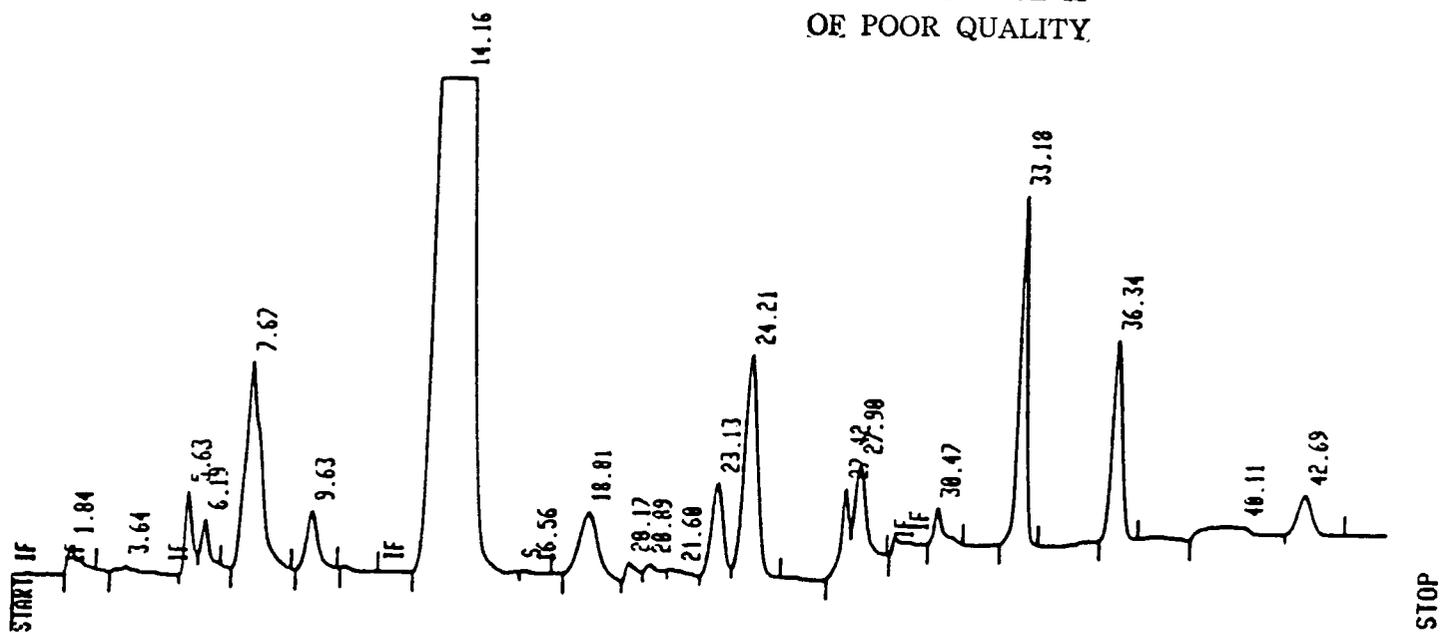


Figure 8-5.- Fixed 24.5 hours after mixing cells and beads.

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RUN # 177
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ESTD

RT	AREA	TYPE	CAL#	AMOUNT
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5.63	622690	PY		0.841
6.19	415010	VB	1	0.481
7.67	3570400	PB	2	4.420
9.63	723800	PB	5	0.716
14.16	2.5584E+08	SBB	7R	277.780
18.81	1462200	BP	10	1.665
20.17	238530	PY		0.322
20.89	251670	VV		0.340
21.60	299460	VV	11	0.324
23.13	1363600	VV	12R	1.606
24.21	3478200	VB	13	4.049
27.42	723190	BY	14	0.970
27.90	1358600	VB	15	1.854
30.47	378250	BB	16	0.425
33.18	2998800	PB	18	2.926
36.34	2213000	PB	19	3.776
40.11	707250	BY		0.955
42.69	748720	VB	21	1.017

TOTAL AREA= 2.7777E+08
MUL FACTOR= 1.0000E+00

Figure 8-6.- Amino acids analysis (HPLC) of serum-free medium (no cells present).

PLANT CELL TECHNOLOGIES IN SPACE: BACKGROUND, STRATEGIES, AND PROSPECTS

A. D. Krikorian, Department of Biochemistry
State University of New York
Stony Brook, New York

H. W. Scheld, PhytoResource Research, Inc.
College Station, Texas

INTRODUCTION

This section has two points of origin. The first, and earliest, is the long-time effort on the part of many workers in development of methodology for in vitro culture of plant cells or parts, and more specifically, the efforts aimed at understanding how plant cells in culture may be induced to produce chemical products of interest and value. The second point of origin is a recent effort on the part of the writers to develop a system of flight experiment hardware which will support basic science objectives in plant or animal cell culture experiments. These two lines of work have now begun to converge: plant culture technique has progressed to the point that we are able to manipulate cultures to obtain meaningful yields of secondary metabolites; the optimal culture vessel for plant cell experiments approaches the size of a small cell culture bioreactor. The stage is thus set for a serious investigation of the production of plant cell-derived biologicals in space-borne manufacturing facilities.

In this paper, we have attempted to summarize the work which has led us to the present state. We first consider the evolution of concepts and then the general principles of plant tissue culture, and finally, the potential for production of high value secondary products by plant cells and differentiated tissue in automated, precisely controlled bioreactors. The literature on plant tissue culture is voluminous; no attempt will be made to recount in detail. Rather, the general course of its development will be highlighted to convey to the reader a feeling for the major milestones in the path to modern tissue culture as well as the difficulties which had to be overcome. In order to maintain rapid flow of the narrative, specific literature citations will not be included in the text. However, a large bibliography of pertinent and current references is included at the end of this paper.

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FOUNDATIONS OF CELL CULTURE

The Development of the Modern Period

The modern period of plant cell culture began around the turn of the century with attempts by Gottlieb Haberlandt to culture plant cells in isolation. Haberlandt, who was destined to become one of the leading botanical investigators of the 20th century, clearly saw the potentialities inherent in culturing isolated plant cells, but also came to realize that it was necessary to know more about the specific factors required for plant cell division. Because of his lack of success, Haberlandt turned his attention to the study of requirements for cell division. Nevertheless, Haberlandt's paper describing his experiments contains numerous points of principle which recur again and again in any consideration of growing cells in vitro. Other workers contributed importantly to the accumulating knowledge. Erwin F. Smith, the great pioneer of plant technology, saw the possibilities of the application of the aseptic culture methods to the study of relationships between a pathogen and its host as that exemplified by the crown gall tumor system.

A number of workers contributed to the technology through investigations of intact organs, notably roots, in culture. Prompted by contact with B. M. Duggar, a pioneer in plant physiology at Cornell University, first Lewis Knudson, then William J. Robbins, exploited aseptic culture methods in their work on root systems. Knudson utilized aseptically cultured roots to study carbohydrate metabolism, and Robbins from this starting point developed his approach to the culture of roots. These root cultures, however, represented organ cultures and there is a basic difference between the growth of an organ culture, such as that of an excised root in which the root apex is already established as an organized structure before culture has been started, and the culture of isolated tissues or of undifferentiated cells. Nevertheless, the countless investigations which finally established the nutritional and environmental conditions for unlimited growth of selected excised root tips, stem tips, and "tissues" of root and stem, have invaluablely aided all those who have attempted to culture other plant materials aseptically for the first time.

Early work with plant "tissue culture" suffered from a number of deficiencies and blind alleys, and much of the early work tended to be tangential to the main thrust. If one uses the early definition that plant tissue cultures are "aseptic preparations of somatic cells and tissues which grow and function without serious physiological derangement, but which at the same time, may not differentiate into distinct organs," Gautheret, Nobecourt, and White probably had the first true cultures which fulfilled this definition. Indeed, during this early period the single criterion of a successful tissue culture was the unlimited capacity for undifferentiated growth. There would

be no point in citing all the pertinent literature of this period, but the well-known works of Philip R. White and Roger Jean Gautheret, which concentrated on the methodology rather than the solving of problems, need not be mentioned, for they developed techniques even though they did not lead to many new concepts. In fact, the unfortunate controversies between White and Robbins tended to focus attention upon the then incompletely understood minutia of technique to the exclusion of the large problems that could be solved by the use of the tissue culture procedures. Similarly in France, Gautheret and Nobecourt became preoccupied with the idea of continued and indefinite cultivation of plant parts and their organs and pursued this line of work even though many of their continuously cultured strains probably deviated very considerably from the tissues from which they originated. Moreover, the methods as developed and adopted by the early workers, such as Gautheret and White, had severe limitations in that they were restricted by the use of large explants on semi-solid media (usually agar). They also seemed concerned with tissue survival and continuous growth by means of callus subculture to the extent that few attempts were made to initiate fresh cultures once a given plant was successfully cultured.

Another very real problem with the technology, as it was then practiced, was that the very definition of a plant tissue culture dictated that it should not undergo organization. This is the very antithesis of what is commonly aimed for now and reflects another of the early limitations.

Plant Cell Culture as a Tool

A turning point came when plant physiologists began to use the tissue culture technique not as mere means of growing biological material, but as a means of solving problems. And at this point radical changes in the design of tissue culture strategies occurred. Notable in this connection were the methods developed in the laboratory of F. C. Steward, then at the University of Rochester. As part of the experimental design to study salt and water uptake during growth of plant cells, he recognized the need to have a system in which he could control or initiate cell division. In the course of routinely screening a number of tissue culture systems for potential adaptation to these experimental ends, beet, carrot, and Jerusalem artichoke tuber explants, potato tuber explants, the inner epidermis of bean pods, tissues from the floral receptacle of crab apple, and ovary wall of cherry and basal meristematic areas of Narcissus leaf and even pistils from unopened flowers of may apple (Podophyllum) were tested in traditional "Latin-square" experiments against a number of potential sources of stimuli such as indole-3-acetic acid, traumatic acid, phenobarbital, 2, 4-D, extracts of fish eggs, yeast extract, stilbesterol (estrogen), and last, but not least, the liquid endosperm of coconut (coconut water, often erroneously referred to as coconut "milk"). The use of coconut water had been suggested by the work of

Van Overbeek, Conklin, and Blakeslee who had shown that heart-stage embryos of Datura that would otherwise abort could be grown to maturity in aseptic culture, using the liquid endosperm of coconut as a supplement to an otherwise standard culture medium.

The work of Blakeslee and Van Overbeek led to an early attempt to find out whether coconut water contained any unique growth promoting substances. Van Overbeek et al. had recognized both heat stable and heat labile substances that had growth-promoting qualities for embryos, and they even recognized a substance that caused embryos to proliferate rather than to grow in an organized way, but attempts to isolate such substances were unsuccessful and led to the notion that growth promoting activity was due to the balance of ordinary known organic and inorganic nutrients rather than to the presence of any exotic and hitherto unidentified substances.

The growth response obtained by Steward and Caplin to the chemical stimuli tested ranged from none or negligible to dramatic, in the case of carrot in the presence of coconut water. It was soon realized, moreover, that coconut water was only one of several fluids (e.g., chestnut (Aesculus) and black walnut liquid endosperm, extracts of the female gametophyte of Ginkgo, etc.), being extracts of nutritive fluids for immature embryos, which could produce the same effect. Therefore, cell division factor(s) were not specific to coconut water, but it could not be replaced by any of the wide range of known substances. It did not take long to establish the viewpoint that a number of substances which act in combination was involved -- even to the extent of appreciating a balance between promotion and inhibition. This philosophy involving the chemical basis of growth regulation in higher plants found expression in the aphorism that "no single substance unlocks the door to cell division" and is a view that has maintained itself even as it has become amplified in recent years.

A desire to better understand the chemical nature of, and even to isolate the stimuli for cell division that resided in the naturally occurring fluids led to the refinement of the growing methods and to the eventual development of a bioassay in which uniform growth of fresh tissue explants was promoted in liquid culture. The difficulties of liquid culture were surmounted by culturing the explants in tubes which were rotated slowly (1 rpm) about a horizontal axis, so that they were alternately bathed in liquid and exposed to air. Using explants of carrot root phloem, 4 mgs in weight, with the inner tangential surface of the cylinders one millimeter from the cambium, Steward and Caplin noticed that there was about a 4-day lag after culturing, followed by a very rapid growth rate reaching a compound interest rate of increase in 12 days of 34.1% per day. This amounted to an increase in cell number from about 25,000 to a million and a half in 8 to 10 days! With a system such as the one described, they could study not only the growth promoting, but also the growth depressing activities of a number of substances. Since the

coefficient of variability of the explants was so low even after a prolonged period of growth, the carrot root phloem system was, and still is, used as a bioassay with wide application. The technique was, however, not without its critics who unfortunately failed to recognize the importance of the size of the initial explants in determining growth rates.

THE TECHNIQUES OF PLANT TISSUE CULTURE

Free Cell Culture

Experiments with explants in liquid culture led to a fortuitous discovery. In un replenished cultures and at the end of about 21 days, the cells in the explants are no longer rapidly dividing and the typical sigmoid growth curve begins to level off, but some of the peripheral cells begin to break away from the parent explant and are freely suspended in liquid medium. Free cells of carrot so liberated in a liquid medium could be transferred as suspensions to fresh media and their growth as free and suspended cells and clusters ensued even in the absence of the parent explants. This technique represented a marked departure from the earlier methods which utilized free cells isolated as such directly from the tissue of their origin or depended on the disruption of proliferated callus cultures by vigorous agitation on rotary shakers. By causing a tissue explant to grow and proliferate in a liquid medium, Steward and his co-workers provided the environment in which cells could rapidly grow and divide.

Muir et al. had published the first successful attempt to culture individual higher plant cells derived from agar grown callus cultures that had been disrupted in a liquid medium. Single cells so isolated could be induced to grow by placing them on a "nurse" tissue, provided in the form of a vigorously growing agar culture, from which it was separated by a piece of filter paper. This ingenious technique provided the milieu in which single cells could proliferate and form large calluses. But it was neither easy to follow their development nor convenient for growing particularly large numbers of cells. The batch culture methodology, made feasible by directly initiating cultures of carrot in liquid medium, provided the point of departure for rapid developments, and for some time, the growing of single cells in microchambers became obsolete.

Somatic Embryogenesis in Free Cell Culture

It was early recognized that small carrot root phloem explants only very rarely formed roots in culture, but carrot cell cultures in liquid media formed roots much more frequently. Suspensions of such strains of carrot

cells capable of forming roots were regularly maintained in liquid by continued transfer of small aliquots, but shoots were never observed. When such cultures, bearing roots, were transferred to a semi-solid agar medium, the first shoots and even complete plantlets were obtained.

The aspect of the work that was recognized at the time as being the most significant was that free cells derived from small explants of mature carrot root phloem and in the absence of a cambium, had been induced to grow rigorously in the presence of a natural fluid. And that after substantial number of subcultures, using sparse and dilute inocula, these could readily give rise to plantlets. When such carrot plantlets were later grown to maturity, and after the appropriate biennial growth period, they flowered. The flowers were normal by all visible criteria and contained normal, viable embryos. It then became possible to raise several "generations" of carrot in which the normal reproductive part of the life cycle was entirely by-passed, although the successive generations were limited through cells derived from the phloem of the mature storage root. By explanting plugs of secondary phloem and culturing them, free cells were again obtained which gave rise to rooted nodules which in turn gave rise to new plants. These observations emphasized that a full complement of genetic information must reside in cultured cells, i.e., that the cells were totipotent. Thus, the old idea that genetic material was permanently altered or lost during the process of differentiation had to be discarded, at least in the case of carrot.

The events which led ultimately to controlling the cultured carrot cells and tissues to express their "totipotency" are well documented in the papers of the period and need not be dealt with in detail here. It is in this period of development of modern culture methods that efforts became increasingly canalized towards the study of morphogenesis in cultured tissues. The work of Skoog and his colleagues, dating back to the mid-1940's, is of special significance. Work on the controlled differentiation of tobacco tissues, first from stem explants, and later from callus derived from pith, took a dramatic turn with the discovery that preparations of autoclaved herring sperm DNA could stimulate (in association with the auxin indole-3-acetic acid) cell division. A very active cell division substance named kinetin, N⁶-furfurylaminopurine, was isolated from such preparations.

The importance of the discovery of kinetin, although it has never been shown to be a naturally-occurring component of plant cells, cannot be overemphasized. It provided the first chemically identified cell division substance. It also led to the dramatic discovery that in combination with auxin, and at least in the case of tobacco, it could either stimulate undifferentiated callus growth, or could lead to the formation of shoots or roots from such callus depending on the relative ratios of exogenously supplied auxin: cytokinin. Higher auxin relative to cytokinin yielded roots; higher cytokinin relative to auxin yielded shoots.

Although we now know that the controls of differentiation and organogenesis are much more complicated than the tobacco pith tissue system implies, the almost simultaneous discovery of the chemical nature of the "first" plant cell division "hormone" and somatic embryogenesis in carrot served as a tremendous impetus to the field of plant tissue culture. The stage was set for a new era in experimental embryogenesis and the study of growth and organizational control in explanted tissues.

Chemical Growth Control for Plant Multiplication

The early investigators working with callus and suspension cultures fully appreciated the significance of their work for multiplication. On more than one occasion it was suggested that mass cell culture methods could perhaps be used for clonal multiplication of horticultural specimens with particularly attractive qualities. Although some efforts were made at implementing culture procedures with practical ends, it became clear that the systems were generally too uncontrollable or seemingly restricted to too few plant species for use on a truly technological level.

Following on the work of early investigators, a great deal of work has been done on evaluating the usefulness of chemically controlling organized growth from a wide range of plant materials. One can reduce the various kinds of observations made on responding systems rather conveniently, however. These responses when seen from the perspective of multiplication, clonal, or otherwise, now permit deliberate strategies to be adopted in the laboratory environment. Table 9-1 lists the bulk of these strategies. It will be apparent that the various routes are largely the outcome of an appreciation, however superficial, that (1) organized growth or indirect organogenesis can be either "husbanded" or fostered in callus systems more or less de novo; (2) organized growth or direct organogenesis can be generated from excised organ or tissue systems without an intermediate and extensive callus stage; (3) somatic embryogenesis can be stimulated to express itself from competent cells, however they may be generated and selected for; (4) various levels of branching or shoot development can be facilitated by releasing certain correlational controls normally present in the intact plant body; (5) plantlets can be generated directly from organs of perennation sometimes precociously inducible in vitro; (6) micrografting procedures performed in vitro can effectively lead to more plants in some cases; (7) ovule culture can rescue or lead to plants that would otherwise be lost; (8) embryo culture can do the same; (9) mega- and microspore culture can similarly yield materials that reflect either of the female or male genotypes, respectively; and (10) infection with a crown gall plasmid genetically altered to stimulate teratoma-like tumors can lead to rescue of multiplied materials.

TABLE 9-1. STRATEGIES FOR MULTIPLICATION OF HIGHER PLANTS IN VITRO

Shoots from terminal, axillary or lateral buds

- apical meristems (no leaf primordia present)
- tips (leaf primordia or young leaves present)
- buds
- nodes
- shoot buds on roots

Direct organogenesis

- adventitious shoot and/or root formation on an organ or tissue explant without an intervening callus

Indirect organogenesis

- adventitious shoot and/or root formation on a callus

Somatic embryogenesis

- direct formation on a primary explant
- indirect formation from cells grown in suspension or semi-solid media

Direct plantlet formation via an organ of perennation formed in vitro

Micrografting

Ovule culture

Embryo rescue

Mega- and microspore culture

Infection with a genetically altered crown gall plasmid

These topics are not as peripheral to the subject of this paper as it may seem because cells can be the starting points to generate plantlets de novo and then via a combination strategy, the techniques outlined in table 9-1 can be brought to bear on the propagules according to a defined need. Even so, no purpose is served in extensively discussing each of these strategies here. Many comprehensive works are now available which address the specifics. Several are cited in the extensive bibliography at the end of this work.

Hormones in Multiplication Technology

Of more direct concern to the subject of bioreactor culture technology is the rationale for hormone application that underlies the currently used strategies for somatic embryogenesis and for induction of adventitious shoots and roots on such unorganized callus cultures as tobacco. It is a strategy widely used commercially in the multiplication of plants from preformed or organized propagules, and it is understood in relatively simplistic terms.

It stands to reason that good familiarity with the biology of any given plant is the first of several prerequisites to successful manipulation in aseptic culture. Botanists appreciated, long before axenic culture techniques came into laboratory practice, that the development of the higher plant body involves the suppression of many, and the development of relatively few, actual or potential primordia. Production of new growing regions, tissues, organs and even new plants were clearly held in check by correlative influences and inhibitions. Applied auxin in the form of indole-3-acetic acid or indole-3-butyric acid was early used to induce root formation on cuttings ever since auxin was chemically identified.

The discovery in the 1960's that explanted shoot meristems and shoot tips of the orchid Cymbidium grown in aseptic culture produce protuberances which resemble normal protocorms and can grow into plantlets provided the most dramatic impetus for the further development of procedures for multiplying and maintaining plants in aseptic culture. Shoot tip and meristem-containing cultures from many other plants have since been exploited in the obtaining, maintaining, and multiplying of stocks. In some cases, a single plant is generated from one cultured shoot tip or lateral bud explant while in others, multiple shoots can be stimulated to develop.

In each of these cases, it is often helpful to force precocious (multiple branches usually in numbers greatly in excess of normal) axillary branches to form, so these can be separated and rooted. Axillary shoots or laterals, can produce additional axillary branches, theoretically in perpetuity, as each newly formed shoot or node explant is subcultured. The method has been applicable to a great variety of species ranging from herbaceous foliage plants to bulbous monocotyledons, and also to woody species.

Micropropagation was originally defined as any aseptic culture procedure involving the manipulation of plant organs, tissues, or cells that produces a population of plantlets bypassing the normal sexual process or non-aseptic vegetative propagation. Stem tips and lateral buds have been, to date, the most commonly used starting point or origin of the primary explant. For this and other reasons, the use of callus, free cells or other of the more demanding approaches covered in the earlier section, rarely comes to mind when the word micropropagation is used. The key feature of micropropagation via

precocious axillary branching, however, directly arose out of the early work on kinetin. Wickson and Thimann were taken by the possibility that kinetin might well exert an effect on the development of buds, not just their initiation as had been suggested by the work of Skoog and Miller. The essential point is that cytokinin can antagonize the inhibitory effect of auxin on lateral bud elongation. One is essentially releasing the tendency for a shoot apex to exhibit apical dominance over the lateral buds.

Murashige and others have found it helpful to segregate the sequence of events associated with the multiplication process as follows:

Stage I - the initiation or establishment stage. Here the initial or primary culture is established.

Stage II - the shoot multiplication or multiplication stage. Here the goal is to multiply shoots.

Stage III - the rooting or pre-transplant stage. Here the goal is to produce a self-sustaining plant that can survive transplantation to soil, greenhouse, or growth chamber condition.

Frequently, specific media or aseptic culture conditions are associated with each of these stages and can be helpful in developing a strategy, or attempting interpretation. However, one ought to avoid the implication that these stages are always temporally discrete and separable. They may not be. Murashige initially reduced the stages of *in vitro* propagation to three: I, II, and III. But a fourth stage, IV or final transfer to the natural environment stage, is also seen as an integral part of the procedure as well. Moreover, an initial stage "0" has also been added. This stage "0" involves selection of the mother plant and selection of a program of pretreatment to render the strategy to be adopted workable.

The fact that many papers have been written on each of these stages or features of *in vitro* multiplication should be indication that the approach is now well established.

Multiplication Via Organs of Perennation Formed in Aseptic Culture

Some plants form organs of perennation *in vitro*. When this occurs, one has the means for multiplication at another level and it may well turn out that direct planting or germplasm storage of plants can be implemented by this means. Potatoes can form miniature tubers, gladiolas can form cormlets, bulbils have been encountered in certain lilies, onion, narcissus, hyacinth, Dioscorea, etc. and, of course, protocorms are produced by orchids. None of

these, other than the orchid protocorm system, has been so controllable that it has been seriously adopted as a means of multiplication.

Micrografting

Micrografting is perhaps best seen in the context of providing virus-free or virus-indexed material for further conventional multiplication. In Citrus, the technique involves grafting, in vitro, of a shoot tip, usually with two leaf primordia, onto a disease-free (zygotic) seedling root stock. Because Citrus seedlings are generally free of virus and hence are used as the stock, and because the shoot apex of the stem which is to be grafted onto the root stock is presumptively pathogen-free, the system offers a unique opportunity to produce mother block plants which are virus-free. It is not particularly important to have high efficiency in the micrografting since conventional grafting can be carried out once specific pathogen-free mother plants are generated. It is standard practice to re-graft onto indicator plants for evaluation of cryptic viruses prior to final large scale nursery grafting in the field. Micrografting has been carried out with such commercial species as apples, camellias, and cinchona, as well as the citrus indicated above.

There is no evidence that exogenous growth regulators above and beyond those used in maintenance of shoot tip cultures play a major role in the micro-grafting technique.

Embryo or Spore Culture

It was shown as far back as the 1920's that one could sometimes stimulate growth, of certain embryos otherwise unobtainable or erratic, in aseptic culture. In some cases, embryos with poorly developed food reserves do not germinate because they are very dependent upon external nutrient sources. For instance, the seeds of orchids contain a very small embryo comprised only of a simple mass of several hundred cells. The embryo is totally dependent, for germination, upon exogenous organic foods such as sugar. In nature, this sugar is provided by a symbiotic mycorrhizal relationship. Another well known example of failure to germinate involves the formation of inhibitors in the seed. Here, embryos can often germinate only after an appropriate period of dormancy. In some plants (e.g., Iris), one can eliminate both the dormancy requirement and the effect of germination inhibitors present in the seed of some hybrids by excising embryos and rearing them in aseptic culture until they reach a size sufficient for transplanting to soil. Aseptic culture has become a widely used and routine procedure for rescuing embryos that would not normally grow into plantlets. In the strict sense of the word multiplication, this is not truly a process of multiplying stock, but considering

that germplasm would otherwise be lost without the technique, then it is appropriate to include it in the list of strategies for multiplication.

The late Emerita de Guzman and her associates at the University of the Philippines at Los Banos had considerable success in the growing of the coconut sport called "Makapuno". Under natural conditions, the endosperm of "Makapuno" seed rots and hence deprives the developing embryo of necessary nutrients. But by removing individual embryos from the seed and providing them with an appropriate nutrient medium, they were able to rescue (and grow seedlings) to a size sufficient for field planting.

Splitting embryos longitudinally is yet another possible means of multiplication. In this case, the plant "halves" are identical, or clonal, of course.

In certain orchids, seeds can be aseptically germinated, and these in turn may develop even in the absence of exogenous growth regulators into protocorm masses, which can be subdivided and multiplied. Populations may thus derive from a single embryo and this is, of course, a clonal population. In cases such as the rare epiphytic or endangered terrestrial orchids, where only seeds may be accessible, this is a reasonable strategy for clonal multiplication.

Rearing of ferns from spores sown aseptically on nutrient medium has been carried out for years as a way of increasing success in multiplication. In recent years, prothallia have been generated from spores and these divided to provide a source of gametophytic tissue. This gametophytic tissue, in turn, can be broken down using extreme procedures such as chopping in an electric blender, and in time sporophytes are formed. Ferns have long been favorite subjects for the study of apogamy - i.e., the development of a sporophyte from gametophytic tissue. What is significant from the perspective of this treatment of growth regulators in tissue cultures is that all this is achieved without use of exogenously supplied hormones.

Androgenesis, or the production of plantlets from anthers as sources of haploid cells has been recognized since the late 1950's - early 1960's. Cultures can be initiated from anthers containing immature pollen grains - actually microspores - prior to the development of pollen grain or mature male gametophyte. In tobacco, for instance, the vegetative nucleus divides to give rise to the proembryo while still within the original wall of the pollen grain. In still other cases, it has been possible to induce isolated pollen grains to form somatic embryos. Plants produced by these means are likely to be dissimilar to their parents and, therefore, the means is not strictly applicable to clonal multiplication, but there are some instances where this indeed results in cloning of the individual. As in the case of fern spores, the culture media that are most often used to achieve

adrogenesis omit exogenously added hormones. On the other hand, gynogenesis, the process whereby haploid plants are produced in vitro by induction of haploid tissues from the female gametophyte, usually employs a more complex medium.

PLANT CELL CULTURE FOR PRODUCTION OF HIGH VALUE BIOLOGICALS

The emphasis given above to discussion of micropropagational technology should not be taken to mean that work on cells in suspension culture came to a halt during the period of the most rapid emergence of multiplication strategies using shoot tips, etc. It is true, however, that the greatest amount of publicity was given to them because there was clear-cut potential for short-term commercialization. As mentioned earlier, it was recognized from the outset of plant cell culture studies that there were possibilities for clonal multiplication using free cells. These ideas took time to mature, however, even as the techniques were being worked out. Similarly, it was appreciated in the mid-1950's that plant cells grown in culture might well be usable for production of specific biochemicals (figure 9-1). As a direct consequence of some of the early work, several industrial research programs were initiated to explore the feasibility of growing cells in bulk. As it turned out, however, most of those attempts led to the conclusion that it is not easy to simulate accurately and at will in unorganized cell cultures otherwise organ-specific or tissue-specific biosyntheses. In those days, the controlled course of metabolism and biosynthesis, which is often linked to morphogenetic complexity, severely limited the economic use of unorganized cell culture systems for biosynthetic purposes. The doctoral dissertation work of one of us (A.D.K.) on that precise problem, initiated in 1959, led to the conclusion that our ability to evoke in culture any feasible feature of normal growth, form and composition of cells in situ was very often a measure of our ability to apply external stimuli that could successfully emulate the genetically based signals that cells must receive during their organized development.

Because the yields of components producible by cultured cells were relatively low when compared to the amounts extractable from intact plant parts, attention was necessarily diverted by most investigators interested in this field to other, more approachable, fundamental problems of differentiation and growth. The advances in our understanding of plant cell nutrition, cell division, differentiation, biochemistry and biochemical genetics has once again made the field approachable and there has been a dramatic rise in resumed interest. The theoretical possibility of using virtually any plant part to generate competent or totipotent cells equivalent to the zygote from which they were derived by a series of equational divisions, makes it all the more reasonable to believe that such cells should contain the full biochemical capacities of the whole organism, if these can be evoked.

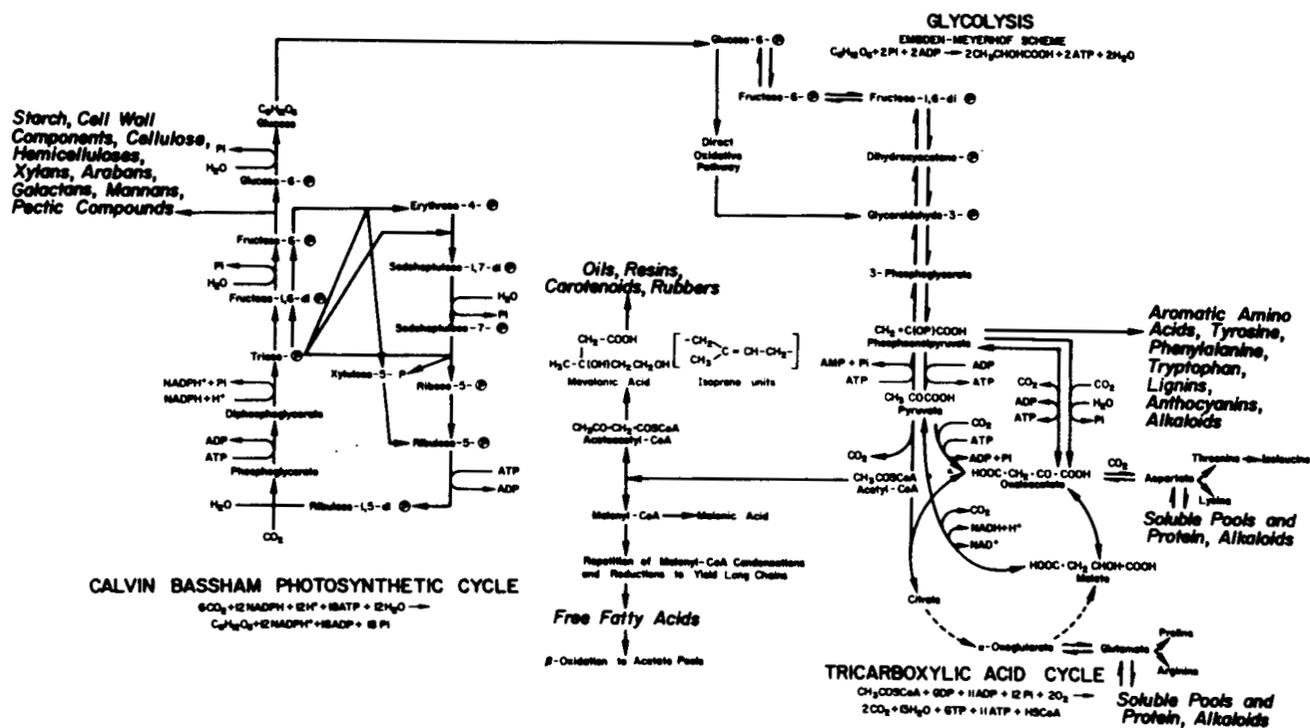


Figure 9-1. General scheme of higher plant metabolism showing biosynthetic pathways leading to secondary product synthesis and accumulation. Note the tight linkage of secondary product synthesis to primary functions.

The aim of much research has been to determine and define those conditions which permit cultured higher plant tissues and cells to express their innate biosynthetic potential. Strategies have included selection of plant species that can serve as models with which to assess the range of problems that must be addressed before full and routine exploitation of plant cell systems can be achieved for specific biosyntheses. One of the strategies adopted has concentrated on the control of, or release of, the suppressed biochemical potential without the need to do this via a complete new ontogenetic cycle of development.

Selection of particular genotypes or "chemovars" of plants intended for culture has been more carefully addressed than ever before. It is well recognized that strains within a species may vary widely in their inherent capacity for production of secondary compounds. There is little hope for achieving high level synthesis from a system limited by its genetics and heritage. Efforts have also been made to decide which plants are best investigated or justified from an economic view. Some estimates have been made that suggest no product producible by cell culture technology is ever going to be economically viable unless it can sell for at least \$80 a gram or \$36,320 a pound. Other estimates are considerably lower, but it is clear that such products cannot be inexpensive.

The availability of appropriate cell lines which are producers is, of course, central to the entire theme. It has long been recognized, however, that procedures must be available to maintain cell lines in a culture bank, either lyophilized or cryopreserved, if industrial application is to be a reality. As it turns out, and primarily as a result of a research initiative directed at maintaining genotypically stable materials for germplasm storage, dramatic progress has been made in the area of cryopreservation.

The use of biological materials or cell types even more precisely selected from a wider range of possible genotypes is now possible through use of protoplasts produced by techniques of enzymatic digestion or mechanical maceration of plant tissue. Such cells may be "plated" out on selective media or under selective environmental conditions in much the same manner as microbial cells. Immunofluorescence-based and monoclonal antibody procedures can also play a role in facilitating the selection of appropriately "turned-on" cells. The rationale here is that those cell cultures which have derived from cells which are not appropriately "programmed", are not going to perform, whereas those that are programmed can be rescued from a larger population of heterogeneous cells, as in a leaf where many of the individual cells having potentially useful characteristics are never "allowed" to express themselves.

In still other cases, brute force has been the instrument whereby cell lines have been screened for production potential. This is easier to do if the substances sought are colored, or fluorescent or have other readily recognizable characteristics. Long-term strategies adopted by Japanese industry to analyze production of shikonin, a red dye from Lithospermum erythrorhizon used in pharmaceuticals, involved such varied approaches as optimizing environmental conditions, selection of high-producing strains, derivation of variants, addition of elicitors, addition of precursors, testing biotransformation and morphological differentiation and immobilization of cells. In the final analysis, the production involved a two-stage process. The first involved growth and the second involved transfer to a setting in which the medium was changed to permit synthesis of the product. This first commercialization, albeit on a small scale in comparison to those routinely used by the antibiotic or fermentation industries, is rightly seen as a pioneering step and is sure to have a heuristic effect on the field.

Additional strategies to stimulate production of secondary substances and metabolites include utilization of the principles of stress physiology as a probe to understand and manipulate intermediary metabolism. These biotic elicitors are an excellent means of confirming the biosynthetic potentialities of cultured plant cells. There is, therefore, an increasing body of literature and knowledge which shows that in select circumstances, and with a great deal of patience and work, sufficiently controllable systems can be achieved for industrialization of higher plant cells in culture.

Certainly, problems remain. Higher plant cells grow relatively slowly in culture in comparison to microbes. Subculture periods are much longer. The secondary products are generally not produced in logarithmic phase, but in stationary phase. Also, products are not generally excreted into the culture medium. Cells must be extracted to yield products. Whereas cryopreservation methods are being worked on and progress is good, we are nowhere near the level of competence reached by industrial microbiology in terms of the technology. This is all the more worrisome since there is a great deal of evidence that change can and does occur in higher plant cells as they remain in culture. Prospects for genetic engineering of cells to produce substances will be best achievable in those cases where simple enzyme systems coded for by single genes are in question. But the fact is that there are not many known examples of such control of high-value, low market volume products. In all cases, the economics will be the "driver" in addition to the biological and engineering feasibilities.

THE SPACE ENVIRONMENT AS A VEHICLE FOR INTEGRATION OF NEW BIOTECHNOLOGIES

While the problems may be rather simply stated, the operational solutions may be quite complex. The duplication in culture with external stimuli of the genetically-based signals that plant cells use to direct their pattern of growth is more art than science and the technology is still in its infancy. It is likely to remain so unless certain innovations are made in the technology. The only feasible way of defining the nature, qualitatively and quantitatively, of the necessary stimuli and conditions is through tedious trial and error, which at this point can only be accomplished at the expense of considerable time and skilled manpower.

An innovation necessary to exploit the full potential of the cultured plant cell is an apparatus capable of large scale screening and automated testing of plant cells against complex programs of exposure to chemical and environmental regimes. There are good reasons for planning at the outset to utilize the space environment as an adjunct to the system. In a micro-g or hypo-g space environment where there is no buoyancy, no convection, no stratification of layers, and where surface tension dominates, we can anticipate major impacts on metabolism that will be reflected in biosynthetic potentials of cultured cells and protoplasts. There are also significant advantages of such a system for a 1-g micro-environment. For example, (1) cell cultures can be grown and maintained under controlled conditions with respect to nutritional and environmental requirements. Such a situation would allow establishment of conditions for optimal cell growth or maximum secondary product formation, and for the selection of high producing genotypes; (2) the cell culture methods would permit location of production facilities in any place without dependence on a region with certain anticipated or required climatic conditions; (3) cultured cells would allow biochemical

production to occur around the year in a reliable manner without interruptions due to agronomic practice, to season, or to other environmental factors or even political factors; (4) biomass production by cells in rapidly growing cultures can be considerably more than in cells in situ; and (5) production in cell suspension culture should be automatable and this can lead to a significantly improved biotechnology; it also provides the basis for disclosing principles which can lead to a still fuller understanding of the entire process of growth, metabolism, and differentiation.

The generally large size of plant cells subjects them to shearing in conventional industrial fermentors. Instrumentation of the sort provided by the bioreactor under development at Johnson Space Center in Houston by Dennis Morrison and his colleagues will provide the means whereby all the technological parameters may be systematically examined with a maximum of precision. We may anticipate relatively few modifications to the apparatus necessary to render it useful to plant cell biologists and biochemists.

We have seen in this broad overview the many possibilities associated with the manipulation of plant cells that are morphogenetically competent. Availability of a bioreactor to generate and grow cells in space and to expose them in a controlled manner and even to generate somatic embryos and plantlets provides a powerful means of providing valuable germplasm for experimentation and for use in a CELSS-type controlled environment life support system. The feature of being a renewable and regenerable resource means that small amounts of initial inocula have the potential to produce huge amounts of material. Having generated plantlets, these in turn provide the opportunity to implement the alternative strategies for multiplication outlined in table 9-1.

One cannot of course reliably predict the outcome of such efforts to draw ever closer the problems of basic plant physiology and biochemistry and those of biomaterials and processing inspace. But one can be assured that anything that facilitates realization of better understanding of controls is sure to pay off. All of the new biotechnologies as they relate to plants depend upon reliable and controllable tissue, and cell and protoplast systems. A bioreactor will go far towards providing a state-of-the-art instrument which can meet the challenge (table 9-2). This, coupled with all the unexpected interrelationships that the space environment can provide and the reactions it can elicit, are sure to give us the wherewithall to move to ever increasing levels of sophistication. It is difficult to conceive of an area of research which can yield as much new and dependable primary data and insight into the inner workings of one of nature's most impressive chemical factories -- the higher plant cell.

TABLE 9-2.- TEMPORAL AND PRIORITIZED RANKING OF RESEARCH OBJECTIVES WHICH
CAN BE SERVED BY AUTOMATED CULTURE APPARATUS

Research with Potential for Near-Term Impact

- Rapid multiplication of select specimens
- Elimination of virus and specific pathogens
- Virus indexing
- Germplasm introduction and evaluation
- Germplasm collection, preservation, and management
- Production of polyploids, haploids, somaclonal variants for new crop production and use in breeding, etc.
- Elimination of certain breeding barriers
 - in vitro fertilization in ovulo
 - embryo rescue and/or storage
 - androgenesis
 - gynogenesis

Research with Intermediate Impact

- All of the above in more recalcitrant species, plus
- Selection for complex traits such as tolerance to stress
 - biotic - diseases and pests
 - abiotic - temperature
 - salt
 - herbicides
 - In vitro mutation breeding
 - Cryopreservation

Research with Long-Range Implications

- All of the above in more recalcitrant species, plus
 - Genetic engineering
 - transformation by selectable genes, etc.
 - organelle transfer
 - wide crosses - somatic hybridization
 - Understanding controls in developmental and physiological processes
-

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POTENTIAL USE OF THE BIOREACTOR TO DETERMINE EFFECTS OF MICROGRAVITY
AND OTHER ENVIRONMENTAL PARAMETERS ON GROWTH OF HYBRIDOMA CELLS

Kenneth D. Ley, Ph.D.
Division of Biomedical Research
Lovelace Medical Foundation
Albuquerque, New Mexico

The monoclonal antibody field is a rapidly growing biotechnology. Monoclonal antibodies have been hailed by many as the perfect medical weapon, the magic bullet, that will be effective against several human diseases, including cancer. Antibodies are produced by the body to defend against foreign agents such as bacteria or viruses. In the past it has not been feasible to treat patients with antibodies taken from the blood of immune individuals because of a lack of suitable donors and the expense of separating the antibodies from other blood components. There is also the risk that some patients will react adversely to these preparations.

Laboratory production of large quantities of monoclonal antibodies can be achieved by hybrid cells called hybridomas. Hybridomas are made by fusing specific antibody producing lymphocytes with laboratory-grown tumor cells, the result of which are cells that have characteristics of both parents. The ability to grow forever comes from the tumor cell--to produce antibody comes from the lymphocyte cell. This technology will allow pharmaceutical companies to make them available to patients.

Although this market is projected to be two billion dollars in diagnostics alone by the end of this decade, the technology has not kept up with demand. According to a recent article in the Wall Street Journal, some projects have made it to the market place, but in general, techniques need to be developed to overcome some of the technical problems. Most hybridoma production to date has been in the mouse system. Production of hybridomas from mouse cells has become almost routine, but the same techniques applied to human cells have not resulted in the consistent production of desirable hybridomas.

In our experience there are essentially five critical steps for producing human hybridomas. These are: (1) stimulation of lymphocytes to produce the specific antibody desired; (2) separation of specific-producing cells from nonproducers; (3) fusion of desired cells with tumor cells by membrane interactions; (4) maintaining sustained production of antibody for months; and (5) separation and concentration of antibody from the cell culture medium. It is our impression, and one held by others in the scientific community, that hybridoma technology is still evolving and significant improvements are needed to increase the efficiency of the steps listed

before we can realize our objective of mass producing monoclonal antibodies for the treatment of diseases.

Research is needed to define more completely the environmental conditions that must be met in order to successfully produce human hybrid cells. We believe the bioreactor being developed at NASA will enable us: (1) to determine the optimal conditions (e.g., pH, O₂, CO₂, nutrients) for growth of hybridoma cells, and (2) to determine whether cell growth and antibody production are enhanced in the microgravity of space.

DESIGN CONCEPTS FOR BIOREACTORS IN SPACE

P. K. Seshan, G. R. Peterson,
Jet Propulsion Laboratory
California Institute of Technology
Pasadena, CA

B. Beard, C. Boshe, and E. H. Dunlop
Washington University
St. Louis, MO

(This paper was previously presented at the Ames Bioregenerative Life Support Systems Conference of Principal Investigators, July 18-19, 1985.)

INTRODUCTION

Bioprocessing in space and in extraterrestrial facilities is both a logical extension of bioprocessing needs on Earth and in some cases a better alternative to obtain biomass and biologicals rapidly and efficiently. In the context of controlled environmental life support systems (CELSS), trade-off studies on food regeneration strategies may point to microbial food production as the choice in terms of efficiency, economy, and fault-tolerance. In addition, their value as redundant and supplementary food sources cannot be overemphasized. Work needs to be done to identify and even genetically modify microbial strains to provide an optimum (70:20:10)¹ mixture of carbohydrates, proteins, and lipids. The promise of high energy efficiencies and weight/volume ratios does provide a tremendous incentive to undertake such work expediently and systematically. While this work is progressing, parallel efforts must be undertaken to address the problems of operating bioprocessing units in microgravity.

Microgravity is not expected to have any significant effect on basic biokinetic rates of biological reactions. However, the associated operations of mixing nutrients/reactants and separating products will greatly depend upon the magnitude of gravitation. These two, in turn, will affect significantly the production rate of the bioprocessing units. Any bioprocessing unit may contain one or more bioreactors. The mixing/separation operations are too complex to model from the first principles. There are no simple correlational procedures to convert engineering data and tools used for terrestrial designs for applicability to microgravity. One can choose between two approaches to arriving at a successful design of a bioreactor for use in microgravity: (1) build right away a candidate bioreactor and associated instrumentation based on terrestrial experience and test it in microgravity. Then conduct subsequent tests to modify the hardware and operating conditions/pro-

cedure to optimize the design; or (2) conduct an experimental program for obtaining key engineering data under conditions of microgravity and use this data base to develop design tools and procedures for the design of space bioreactor systems for a broad range of applications. The authors tend to prefer the second approach as the one providing the most value for the money.

BACKGROUND

A major research thrust of NASA's CELSS program is to develop practical and energy-efficient ways to recycle all of the materials involved in life processes so that a controlled life support system requires as little resupply and energy input as possible to sustain spacecraft crews for long-term space habitation.

Microorganisms as Food Sources

Conventional food sources consist of higher plants and animals. Unconventional food sources for human consumption are photosynthetic algae and bacteria and nonphotosynthetic bacteria, yeasts, and fungi. Conventional food sources are highly palatable, but require long lead times to produce. Under conditions of epidemic loss of conventional food sources, recovery may be prolonged or impossible. The photosynthetic energy efficiency of higher plants is less than 3%. Even though conventional food sources will be our best choice because of our excellent culinary experience with them, they are not the most abundant and dependable in the context of an enclosed extra-terrestrial habitat with conventional food sources, and survival under conditions of "drought" during a long-term space mission can be realistically estimated to be near zero. Microbial food sources such as algae, yeast, and fungi are unconventional and have been considered for use as supplements to conventional food. Their nutritional content based on current data can be presumed to be adequate to meet human dietary needs. Microbial food production systems have the advantages of lower weight/ volume requirements² over conventional plant/animal production systems and they also account for superior energy utilization in the production of carbohydrates from CO₂ and H₂O. A quick summary of what it takes to produce an acceptable menu of food items for space habitats can be found in the literature² and the problems do not seem to be intractable. Therefore, it is not a far-fetched assumption that adequate nutritional consumption can be achieved using microbial food sources and the microbial mass can be made palatable to humans through development of suitable food processing techniques.

Microbial Growth Chambers

There have been efforts made by both U.S. and Soviet scientists to design microbial growth chambers. Two plans have been suggested by Martin-Marietta Corporation.^{3,4} One involves a flow circulation loop and the other is a cylindrical fermenter design. The flow circulation model is designed for both production and collection of cell mass; however, it is more suitable for bioprocessing than cell harvesting. The cylindrical fermenter is more like the standard terrestrial fermenter adapted to accommodate microgravity environments. In the early days of CELSS, the use of hydrogen bacteria as regenerative food was considered.⁵ An apparatus for operating such a system was suggested by scientists working for NASA. The Soviets also did some preliminary work on H₂ bacteria growth in their "flying oasis" which was reported to have flown on Soyuz 13 in 1973.⁶ Both NASA and Soviet flight programs included algal growth chambers which were tested in various stages of development from ground-based studies to flight models.⁷⁻¹⁰ Neither program generated sufficient data to evaluate their progress.

For over a decade, NASA personnel at JSC have directed the development of a bioprocessing system that includes both the production of pharmaceutical products and their separation in space. The bioseparation process has been demonstrated on successive STS missions over the past 2 years. Work in the bioproduction area has not progressed quite as rapidly due to concentrated effort on bioseparation. The project is designed to bring about the culture of mammalian cells to produce pharmaceutical products. Terrestrially, the culture of cells is compromised by sedimentation and oxygen transfer limitations. Microgravity can help overcome these problems. Over the past few years, U.S. and European flight experiments have shown positive microgravity effects on eucaryotic cell growth and cell size. A space bioreactor for cell culture has been proposed for operation in microgravity.¹¹ It is designed for eventual tandem operation with continuous flow electrophoresis. The elimination of sedimentation or bubble buoyancy is thought to aid in the growth and maintenance of mammalian cells which are extremely shear-sensitive. The purpose of these attempts is to enhance the production of pharmacologically important hormones and other medical products. It is important to note that design and performance data from the mammalian cell growth programs are not immediately applicable to the growth of microorganisms. The requirements for microbial food production units in a CELSS environment include high intensity cultures requiring significantly higher quantities of oxygen and mixing rates which would shear mammalian cells.

SPACE BIOREACTOR - DESIGN CONSIDERATIONS

Since the emphasis in this paper is on unconventional food production for CELSS, the following discussion will be concerned with fermenters as micro-

bial growth chambers. Bioreactors for other applications will have many characteristics in common with fermenters and their design and operation can have many similarities to the design and operation of fermenters.

A typical industrial fermenter on Earth will not operate in microgravity for the following reasons:

- Gas bubbles will not rise through the fermentation media due to absence of significant buoyancy forces.
- There will not be a single level separating the gas and the liquid. The gas bubbles may not disengage at these multiple interfaces.
- As gas transfer efficiencies of 100% are practically unachievable on Earth even in the absence of other fermenter constraints, there is not reason to believe that they will be attainable in microgravity. Phase separations are not spontaneous.
- Foaming is likely to be an even more severe problem as surface forces causing foaming will be more predominant in microgravity.
- The fermenter may oscillate between continuous liquid phase and continuous gas phase or both may coexist in various regions of the fermenter volume.

In submerged culture, aerobic microorganisms grow very rapidly until at a critical cell mass they are consuming all the oxygen that a fermenter can supply. For growth conditions of relevance to microbial food production, the biological kinetics are sufficiently fast that the rate of transfer of oxygen and rate of removal of carbon dioxide determine the microbial growth rate. Surface area of gas bubbles and internal convection within air bubbles control the rate at which the oxygen transfers to the growing cells. Buoyancy forces also act to enhance gas transfer by participating in more intense surface renewal and gross mixing. But in microgravity these forces are too small to be significant. Therefore, to provide efficient mixing of gas and liquid inside the fermenter, stable colloidal gaseous dispersions must be generated within the fermenter with the help of suitable surfactants.¹² Even though the surfactants will inhibit the mass transfer rate across the gas-liquid interface, through proper choice on concentration of surfactants it is possible to ensure that the increase in interfacial area more than compensates for the inhibitory role of surfactants. A second approach to overcome the problems of bubble size and lack of buoyancy is not to generate bubbles at all in the liquid medium, but to transfer the gases across a suitable membrane at a rate equal to the dissolution rate of gases in the liquid. A third approach will be to induce centrifugal body forces in the liquid medium and provide for buoyancy forces for gas bubbles to "rise" to the center. As

in terrestrial designs, baffles and other flow redirections can be provided inside the fermenter to augment the mixing intensity.

In microgravity, reluctance for dissimilar phases to separate is a serious design issue. If colloidal gas bubble suspensions are employed, at the end of required cell growth, the surfactants must be disabled without toxicity and detriment to nutritional quality. Separation of gases from liquid and separation of cell mass from liquid will necessitate suitable membrane transfer or centrifugal separation units.

The problems associated with mass transfer can also be expected with heat transfer. Natural convection will be too feeble in microgravity and forced circulation over heating or cooling surfaces will be necessary which is also the preferred approach in terrestrial designs as well.

The problems of mixing and separation in microgravity will have to be overcome in suitable ways depending on the type of fermenter operation. Fermenters can be operated in batch mode, feed-batch mode, and continuous mode. In the continuous mode, the choice is between a single plug flow design and a multiple CSTR design with cocurrent or countercurrent gas flow.

Fermenter configurations vary owing to emphasis on one or more of the following key parameters:

- Oxygen transfer intensity
- Power economy
- Cell growth rate
- Production rate of other products

NEED FOR ENGINEERING DATA

In addition to physical configuration of a space fermenter, a designer must determine quantitatively the following:

- Power required for mixing.
- The space velocity or space-time which are measures of fermenter volume or the amount of time the nutrients and gases must remain in the fermenter for the required production rate of cell mass.
- The mass transfer rate achievable and the associated gas bubble size, mixing intensity, and interfacial area.
- The heat transfer rate necessary to maintain the temperature within the optimum range.

For terrestrial designs, power demand for agitators is determined from a correlation of a dimensionless quantity called power number with the Reynolds number based on the impeller distance. Data for this experimentally determined correlation were obtained in terrestrial agitators. The Froude number (the ratio of convectional acceleration to gravitational acceleration) associated with these data was less than 2 in most cases. This correlation cannot be applied without corrections to conditions of microgravity where the Froude numbers are very large. And the corrections must be experimentally determined.

The size of fermenter volume for a given rate of production of cell mass will depend on the mass transfer rate of oxygen through the liquid film which is determined with the help of the quantity, $K_1 a$, which is a product of the mass transfer coefficient and the associated interfacial area. A number of factors determine $K_1 a$ which include bubble and cell dimensions, fluid density and rheological properties, agitator and fermenter geometry, and power input for agitation. Among these, bubble shape and dimensions, dynamics of bubble movement, and hence, the gas-liquid interfacial area and the agitator power input are affected by the absence of gravity. As pointed out by Oldshue,¹³ $K_1 a$ does not scale in the same way as reactor size and agitation rate do. The design tools involving correlations of $K_1 a$ with the other factors must then be recreated for microgravity conditions.

Only two key parameters in the design of fermenters for microgravity application have been discussed above. The purpose was to illustrate a design engineer's concern in having to use the terrestrial data and correlations to design space bioreactors. On the other hand, it may turn out that for some of these parameters the corrections for microgravity application are indeed small, but it is not possible to know that without conducting experiments specifically for obtaining values of these key parameters in the region of high growth rate of cell mass. It is also necessary to determine experimentally the parametric region where bubble formation, fouling, foaming/entrainment occur under conditions of microgravity.

Once the database for microgravity operation of fermenters is established, a clear and dependable design methodology can be established to design space fermenters for any configuration, size, and product. Even if the database is not extensive, it is a great help to the design process to obtain good estimates of the magnitude of corrections to be applied to terrestrial design data.

TWO CANDIDATE SPACE FERMENTERS

The problem of intimately mixing oxygen with the fermentation broth or separating product gases in the absence of gravity can be overcome by designs

that are "gravity independent." Two such design concepts are presented here. In the first, direct gas-liquid contact is eliminated thus obviating the need for dealing with three-phase hydrodynamics in microgravity. In the second, the required gas-liquid contact and disengagement are forced in a controllable and predictable manner.

The "Gasless" Fermenter

The "gasless" fermenter is a closed sterilizable vessel through which an equally spaced bundle of polydimethyl silicone (PDMS) tubes pass. The PDMS tubes carry the oxygen required for fermentation and the carbon dioxide to be removed from the fermentation broth. Fermentation medium consisting of salts, carbon substances such as sucrose, and vitamins surround the tubes and fill the vessel. The fermentation fluid can be circulated through an external loop or agitated with an internal marine type propeller to keep uniform concentrations inside the vessel.

PDMS is six times as permeable to oxygen as polyfluorosilicone and 25 times as natural rubber and 600 times as high density polyethylene. PDMS preferentially transfer carbon dioxide over oxygen by a factor of 6 to 1. Oxygen diffuses rapidly through PDMS tubes to enter the fermentation broth by dissolution. Silicone hollow fiber tubes are a good choice because they, in addition to permitting high gas transfer rates, exhibit excellent biocompatibility and nonadherence to biological materials as testified by the choice of the same material for human and animal surgical implants. However, due to nonuniformity in tube thickness or tube packing density and in regions of stagnation of the fermentation broth, a small number of oxygen bubbles may form inside the vessel. These bubbles should be not allowed to build up and create problems of direct gas-liquid contact. As a safeguard against this, a small fraction of the fermenter contents will be degassed in a low-speed centrifugal separator and the separated liquid returned to the fermenter.

Carbon dioxide passes even more freely than oxygen across the silicone tube walls. However, the removal rate will also be governed by solubility of carbon dioxide in fermentation broth which again is a function of the pH. Carbon dioxide could be removed from the same tubes containing oxygen, or a portion of the tubes in the bundle may be dedicated for carbon dioxide removal. The use of a carrier fluid such as amines in these dedicated bundles is also a possibility.

Using a 1.5 liter Braun fermenter and a single strand of PDMS tubing, a yeast culture was grown successfully as a preliminary demonstration of this concept at Washington University. Air at 1 atmosphere pressure was supplied to the fermenter through the PDMS tube. The tube outlet was connected to a mass spectrometer gas analysis system which allowed the uptake of oxygen by the fermenter to be measured. The tube occupied 0.08% of the fermenter volume.

Transfer of oxygen into water and a fermentation medium as well as sustained yeast growth were demonstrated. Oxygen transfer rate into water at 37° C was found to be directly proportional to the oxygen driving force. Transfer of oxygen into the fermentation was five times slower than into water. An inoculum of yeast (*S. cerevisiae*) grew to produce a culture with a cell density of about 1g/l in 2 hours. Visual and microscopic examination of the PDMS tubing showed no evidence of fouling after being left in the fermenter for several days.

The "gasless" fermenter was modelled as three CSTR's through which the fermentation broth circulates in cyclic fashion. Through two of these CSTR's PDMS tubes pass carrying oxygen to the broth and carbon dioxide out of it. Monod's model for cell growth was adopted. With this model, a computer program was written at Washington University and the effects of liquid flow rate, gas flow rate, and fraction of total number of tubes dedicated to CO₂ removal. At gas flow rates below 0.5 l/min, dedicating tubes to CO₂ removal actually helped increase the final cell mass. However, above 0.5 l/min of gas flow all the tubes had to carry oxygen to meet the demand for cell growth. Further, it was found that at flow rates of gas above 1 l/min cell yield does not significantly increase. When five percent of the fermenter volume was dedicated to CO₂ removal tubing, a very sizable decrease in dissolved CO₂ concentration was obtained. This result is significant for prevention of bubble formation inside the fermenter.

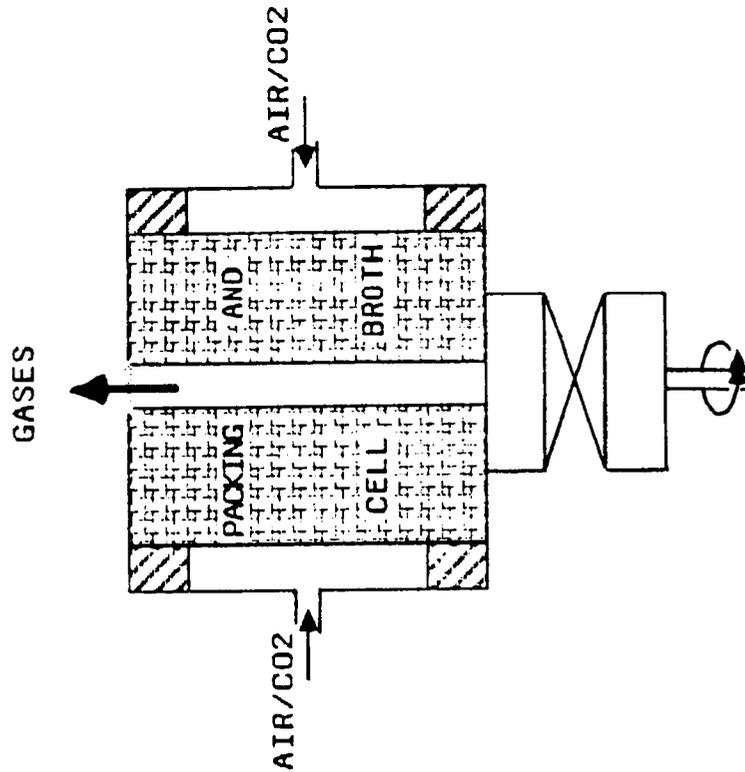
The absence of direct gas-liquid contact is a unique feature of this design concept eliminating problems of three-phase hydrodynamics. This fermenter does not require a gas disengagement volume and since the volume occupied by the silicone tubing is not expected to exceed 10%, volume available for cell culture is 90% compared to 60-70% for conventional fermenters. High oxygen transfer intensities, fewer moving parts, and low shear rates are some of the major advantages of this design. Potential problem areas to be addressed during the design and operation of this fermenter are (1) possibility of membrane fouling, (2) regions of stagnation, and (3) formation of oxygen bubbles.

The Rotating Packed Bed (RPB) Fermenter

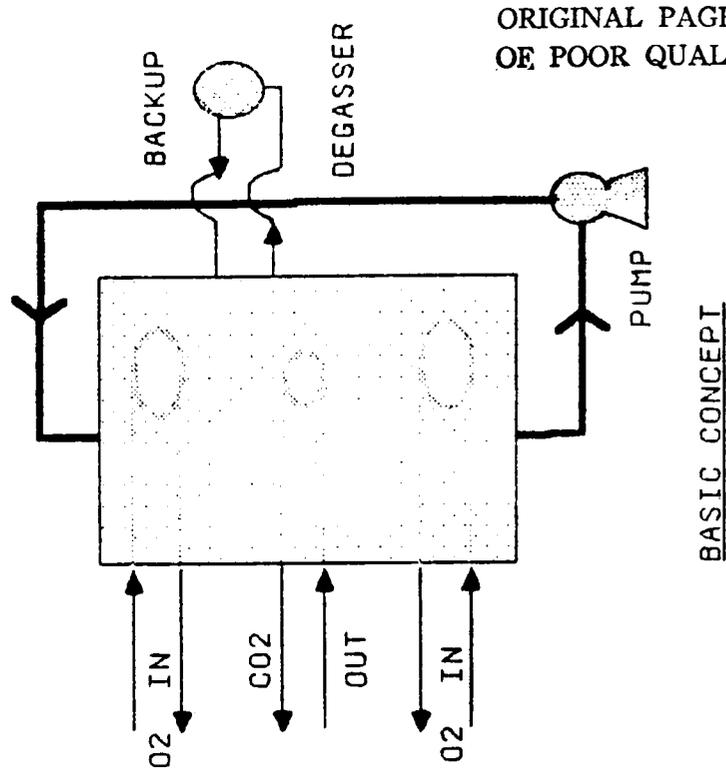
The RPB shown in figure 11-1 has a cylindrical housing and, proceeding inward, has an annular region for air/oxygen distribution followed by a region of small packings and then a central region for entry of liquid and exit of gases. The whole assembly rotates about the axis of the cylinder. The rotation rate can change the throughput rate or, conversely, for a given throughput rate the mass transfer rate can be changed significantly. The RPB fermenter will not run at high speeds associated with the "Higee" units for fear of disintegration of cell mass. However, the packed bed will provide a more intense renewal of gas-liquid interface so that it may be possible to

PROPOSED MODEL SYSTEMS

ROTATING PACKED BED



"GASLESS" MEMBRANE REACTOR



ORIGINAL PAGE IS
OF POOR QUALITY

BASIC CONCEPT

Figure 11-1.- Microgravity bioreactors for CELSS.

forestall any mass transport limitation on overall cell growth which is an important consideration in the design and operation of fermenters. The radial depth of packing will depend on the space-velocity (or space-time) required to achieve a certain growth rate. Since yeast culture is a low temperature operation, fragile ceramic packing need not be used. The packing material selected will withstand the high g's developed during STS liftoff.

The RPB was adapted from the commercial "Higee" unit of ICI Chemicals. This "Higee" unit was used to intensify chemical separations and its operating principles adapted to microgravity use provide a novel fermentation mode that could prove very efficient. Intensification through induced gravity provides a means of dealing with two-phase fermentation. A brief description of the operating principles and how this can be adapted to microgravity fermentation follows.

The Sherwood flooding correlation for packed beds provides a good estimate of the highest gas velocity which can be obtained for a given value of the ratio of liquid to gas flow rates (L/G).¹⁴ The gas velocity (U) appears in the expression as $U^2 a / g e^3$ where a is the specific area of the packing and e is the packing voidage. The term g , which normally represents acceleration due to Earth's gravity, has been recently generalized¹⁵ to mean ambient acceleration to extend the correlation conceptually to other body force fields. When this is done, the correlation opens up new possibilities of packed bed operation through what has come to be called process intensification. For a given value of L/G and the flooding limit predicted by the correlation, by decreasing the specific area of the packing (a), or increasing the packing voidage (e), higher gas velocities could be achieved. However, even the latest improvements in packing design could not dramatically provide increases in gas velocity. Dramatic increases in gas velocity in a packed bed meant correspondingly high shear rates across the gas-liquid interface resulting in a great intensification of interfacial activity. This was indeed absolutely necessary for efficient interphase mass transport. With the announcement of ICI's "Higee" units, the engineering profession became keenly aware of the g -term in the Sherwood correlation which has been taken for granted as a constant to be used to compute the gas velocities correctly. By opening up the possibility that g could be varied by reconfiguring an absorption or distillation unit into a high-speed rotating cylinder packed with much finer packing than could be used before, ICI engineers demonstrated a dramatic reduction in equipment size and weight for a given separation operation. By increasing g in a rotary mode, one had a choice between increasing throughput rates for a given packing size and type or increasing the mass transfer rate by enabling the same throughput rate through much finer packing.¹⁵

The RPB fermenter will not quite operate at the level of intensification achieved by the "Higee" units for two reasons: (1) there is no advantage in

supplying dissolved oxygen any faster than the consumption rate of oxygen by the growing cell population, and (2) very high interfacial shear rates obtained in "Higee" units can damage cell walls and terminate fermentation or cell growth process. However, an optimal rotation rate and radial distance will be adopted to take advantage of the intensification effects of centrifugal acceleration while maintaining healthy cell growth. There is another important difference between RPB and "Higee" units. "Higee" units are very efficient countercurrent gas-liquid flow generators for rapid separation operations. The RPB fermenter is, on the other hand, a fermentation reactor and is not a mere component separator. Therefore, the RPB fermenter is limited in its liquid throughput rate to allow the long space-times required for growth of biomass. In fact, this fermenter may fall on one extreme end of the Sherwood correlation corresponding to very low liquid throughput rates and very high gas velocities.

The "Higee" units in operation either in industry or in the laboratory are exclusively designed and built for separation operations such as distillation and absorption. No "Higee" unit has yet been built and operated as a chemical reactor or fermenter. A RPB fermenter combines gas-liquid mixing, cell growth and gas liquid separation in one operation with the advantages of lower volume and weight requirements compared to conventional fermenter types. Potential problem areas to be dealt with during design and operation of RPB fermenters are entrainment of liquid and foam by the rapidly countercurrent gases.

GROUND AND FLIGHT TESTS

Our approach to developing these bioreactor concepts is to ground-test and then flight-test identical fermenters. Two or more sizes would be initially tested with a view to specifically measuring mixing effectiveness, bubble sizes, mass transfer coefficients, power required for agitation, etc., in addition to growth rate of cell mass. By this approach a sound database is generated, reliable scale-up factors are derived, and procedures for applying corrections to terrestrial designs, so as to obtain designs for microgravity conditions.

The design procedure developed will enable the design engineer to calculate, for a given cell mass production rate, optimum gas velocities in a "gasless" fermenter, optimal rotation rates and radial distances, and intensification factors in a RPB fermenter. In addition, the design engineer will have the information to guide his choice of fermenter configuration, tubing size and arrangement, degassing requirements, carrier fluids, control of membrane fouling, secondary metabolite production rates and their disposal, extent of foaming, extent of channeling of gases, and cell disruption as a function of operating conditions.

Since both designs are essentially gravity independent, their development can largely be done terrestrially. This will provide cost savings by elimination of the need for numerous flight tests.

CONCLUSIONS AND RECOMMENDATIONS

Microbial food sources are becoming viable and more efficient alternatives to conventional food sources especially in the context of CELSS in space habitats.

Since bioreactor designs for terrestrial operation will not readily apply to conditions of microgravity, there is an urgent need to learn about the differences. These differences cannot be easily estimated due to the complex nature of the mass transport and mixing mechanisms in fermenters. Therefore, a systematic and expeditious experimental program must be undertaken to obtain the engineering data necessary to lay down the foundation of designing bioreactors for microgravity. This may be the harbinger of a major sub-discipline called variable gravity process engineering.

Two bioreactor design concepts presented here represent two dissimilar approaches to grappling with the absence of gravity in space habitats and deserve to be tested for adoption as important components of the life support function aboard spacecraft, space stations, and other extraterrestrial habitats.

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CELL CULTURE EXPERIMENTS PLANNED FOR THE SPACE BIOREACTOR*

Dennis R. Morrison, Ph.D.
NASA Johnson Space Center

John H. Cross, Ph.D.
Technology Inc.
Houston, Texas

Many opportunities to study different cell culture technologies and downstream processing will be afforded by a multi-step bioprocessing operation in space. A typical space bioprocess would consist of: (1) a unit to separate the cells that produce a useful biochemical; (2) a bioreactor to culture these cells and isolate the culture medium containing secreted products; and (3) a unit to concentrate and purify the product. It appears that a process centered around the culture of human cells that produce hormones or other macromolecules having clinical use would be the most interesting. The first and last steps have already been demonstrated during experiments on the Space Shuttle. Human kidney cells which produce urokinase and t-PA and pituitary cells which produce growth hormone and prolactin have been separated by electrophoresis on STS-8. Commercial product purification experiments on STS-7, 41-D, 51-D, and 61-B have demonstrated the advantages of the continuous flow electrophoresis for purifying hormone products direct from concentrated culture medium. However, the intermediate step, culturing the cells in a pilot-scale bioreactor remains to be done in microgravity. An approach is presented based on several studies of cell culture systems. Next, previous and current cell culture research in microgravity is described which is specifically directed towards development of a space bioprocess. Finally, cell culture experiments now planned for microgravity sciences mission are described in abstract form.

BACKGROUND

Assessment of Different Cell Culture Systems

Conventional industrial and laboratory methods of growing human cells have been limited to systems which grow and maintain cells in a monolayer. That is largely due to the anchorage dependence of mammalian cells. Virtually all normal human cells (except for a few types) must be firmly attached to some suitable surface before they will proliferate, and eventually secrete

* This paper was supplied as a handout to participants at the Space Bioreactor Science Workshop held at the Johnson Space Center, August 22-23, 1985.

products. Attempts to grow large numbers of cells on the inside surface of roller bottles and in chambers containing multiple plates have enhanced large-scale cultures, but not enough. Some additional success has been achieved by growing cells attached to spiral-wound membranes and to the inner surface of hollow-fiber dialysis bundles, in which nutrients can be supplied through the permeable membrane from media circulated on the opposite side of the membrane or hollow fiber. These techniques have difficulties with oxygenation, and difficulty in maintaining precise control of the culture environment.⁶ Also, problems of cell recovery and cleaning have made large-scale operation extremely difficult and costly. Extensive research and development programs and our own laboratory comparisons have indicated that perfusion-type suspension cultures of mammalian cells in microgravity would be highly desirable from a process control and economic viewpoint.

Some of the major problems associated with monolayer cultures are (1) growth taking place in only two dimensions (surface area), (2) cell proliferation limited by depletion of oxygen and nutrients, (3) difficulties with media circulation and volume-to-cell number ratios, (4) cell/product recovery, (5) cleaning, and (6) sterilization could be solved by using suspension culture. Unfortunately, almost all human cells of pharmaceutical interest grow quite poorly in modern suspension culture systems. Under optimum small-scale conditions, the maximum density of cells is approximately 30×10^6 cells/cm³.¹⁴ This concentration is several orders of magnitude less than the number achieved in microbial cultures. Since the amount of product formed normally is directly proportional to the number of the cells, high cell concentrations are critical to successful commercial cultures.

Currently, 150-180 micrometer diameter carrier beads are used to grow anchorage-dependent human cells in a suspension culture. The beads, with attached cells, are kept in suspension by stirring and the number of cells that can be grown is proportional to the concentration of beads that can be suspended. This can present a dilemma, however, because as the number of beads increases so does the need for mixing to provide nutrients and remove wastes. As the mixing becomes more vigorous, more cells are damaged by shear forces and bead collisions. This has led to development of new, more gentle stirring methods and reactor designs.⁴ The ultimate low shear experiments (using cells attached to microcarriers) can best be performed in microgravity, since mixing is only required to distribute nutrients and wastes. This should lead to increased viability, higher cell concentrations, longer culture runs, and perhaps more product secretions than can be achieved on Earth.

Continual supply of nutrients and oxygen is best accomplished by continuous perfusion of culture medium. Spin filter techniques⁷ have been successful for separating the cells from the spent medium in stirred suspension reactors. However, conventional spin filter systems do not suspend microcarriers well and produce high shear stress to the attached cells. Oxygen is best

supplied by circulating the medium through an oxygenator before it enters the culture vessel. A bioreactor operating in microgravity, however, is expected to offer certain advantages in the precise control of dissolved oxygen. These advantages will be discussed in the experimental abstracts below.

Experience gained from microbial fermentation also has shown that continuous cultures require precise control of environmental conditions. In mammalian cell cultures this control is even more critical. Precise maintenance of shear, nutrients, waste levels, and the microenvironment surrounding the cells also are important design criteria for a space bioreactor.

Previous Studies Undertaken by NASA

Cell culture experiments leading towards a space bioprocess have been indicated since the electrophoresis experiments on the Apollo-Soyuz Test Project (ASTP) in 1975.¹ Electrophoretic separations of intact cells would be the first step in a bioprocess (on Earth, electrophoresis of cells gives poor resolution). It was hypothesized that in microgravity the resolution would improve, because sedimentation and density-driven convection currents would be absent. A successful separation was achieved for human embryonic kidney (HEK) cells, which produce a medically-important enzyme called urokinase. One of the separated cell fractions produced six to seven times more urokinase than ever before possible under commercial tissue culture conditions.²

Urokinase activates plasminogen to produce plasmin which, in turn, dissolves blood clots after they are formed. Pharmaceutical laboratories are using large scale culture of human kidney cells to produce urokinase for clinical use. Since this agent is a protein, it is desirable for immunological reasons to use human sources. Previous laboratory studies have shown that only a small percentage of cultured kidney cells actually produce the enzyme.³ The efficiency of commercial production could possibly be enhanced if the high urokinase producing kidney cells were cultured. Kidney cells are a prime candidate for the first experiments using a space bioreactor.

During Skylab III, cultures of a strain of diploid human embryonic lung cells were studied for 28 days.¹⁰ Comparisons between cultures kept in microgravity and in 1-g showed no detectable differences in mitotic index, cell cycle, or migration. Growth curve, DNA microspectrophotometry, phase microscopy, and ultra-structural studies revealed no effects of microgravity. Although these results suggested that cells are not altered during space flight, more recent results on STS-8 indicate that cell attachment to microcarriers is significantly increased¹⁵ and that hormone secretion from mammalian cells is reduced in microgravity.⁸

In another type of experiment, the attachment of HEK cells to microcarriers was compared in microgravity and on Earth.¹⁵ This was an essential first step for devising a reactor for a suspension cell culture. The cells were still anchorage-dependent in microgravity, and although attachment was increased, proliferation on the beads occurred normally during the first 24 hours.

The basic concepts for a space bioreactor were proposed in 1977 based on extensive experience with terrestrial fermentation systems.¹² One important idea was that oxygen transport would be faster than in microgravity. This was based on the work by Fester et al., who found that surface tension forces insignificant in 1-g become important in microgravity.⁵ A second idea was a sophisticated process control scheme, which would maintain the rigorous environmental control required by mammalian cells, but minimize operator (astronaut) attention. In 1978-79, under contract to NASA, Arthur D. Little, Inc., conducted two workshops to define the scientific principles involved in the production of biologicals from cells in microgravity and to identify specific advantages or disadvantages of attempting cell culture biosynthesis of a specific biological product under microgravity conditions. The workshop participants identified factors that constrained large scale mammalian cultures and selected those that should be relieved by microgravity. They then proposed experiments to explore these factors. The most important categories were

- Shear effects (mixing, flow patterns, gas transfer, etc.)
- Microenvironment
- Techniques to increase cell density

Categories of secondary interest were

- Use of primary cells or cell lines
- Genetic selection in microgravity
- Anchorage dependence in microgravity

Finally, interested participants were asked to propose systems that could be used to develop specific experiments. Pituitary cells were one proposal, which led to the electrophoresis separation experiments conducted on STS-8.

Although cell culture process technology has evolved rapidly, the factors that constrain large-scale culture still apply. At a "Workshop of Bioprocess Scale Up" held in late 1983, participants discussed several of the problems that the space bioreactor is being designed to explore.¹⁴

Studies Now in Progress

The conceptual studies made it clear that the development of a space bioreactor would require a collaborative effort with university and industrial partners. Recognition of this led to the development of a Space Bioreactor Laboratory at JSC. Over the last 8 years, JSC has used in-house research, cooperative research with industry and academia, and symposia to build a body of information pertinent to conducting cell culture and cell biology research in space. The conceptual design for the bioreactor is being reduced to practice now under a plan to flight test the system in Spring 1988. (See figure 12-1.)

The basic design is a perfusion reactor with a new version of a spin filter (Virtis Co.), combined with a stirring vane similar to that described by Feder and Tolbert.⁴ The reactor is designed to operate with no headspace. Sensors for pH, dissolved oxygen, CO₂, and pressure are located outside the reactor vessel before and after the reactor in a medium circulation loop. This provides for differential measurements across the reactor and simplifies control of turbulence and shear within the reactor. Figure 12-2 shows an early version of the bioreactor test unit.

In the cell-free medium circulation loop are vessels for oxygenation, separation of wastes, and isolation of a high molecular weight product. Concentration of the crude product will be required before final purification by CFES. The process control scheme is being modelled. Primary HEK cells attached to microcarrier beads will be used for early runs. They will be maintained on serum-free medium rather than in a growth mode. Reliable assays for plasminogen activators have been developed for kidney cell separation experiments in microgravity.¹¹ Several design criteria are imposed by the design of the Shuttle Orbiter. Compactness, energy efficiency, and a self-sufficient process controller are required. The assembled bioreactor (less the process controller) must be autoclaved shortly before loading cells and installing the bioreactor in the middeck of the Orbiter. This has limited the choices of pump, sensor probes, and other parts.

Various academic research studies have been initiated by the Principal Investigator (D. R. Morrison) to consider the effect of microgravity on the process. (See table 12-1.) The collaboration of Baylor College of Medicine, Rice University, and the University of Houston has been arranged through the new Bioprocessing Research Center at the University of Texas Health Science Center at Houston (UTHSC). This grant also provides for operation of a ground-based CFES unit, for study of alternative separation techniques, studying shear and mixing in the reactor vessel, and developing aspects of the process control system. Some preliminary results on the effect of shear stress on HEK cells have been reported.¹³ The maximum shear force tolerated by the cells was determined as well as the shear range which caused changes

in cell morphology. More interestingly, it was concluded that increasing the shear stress and then lowering it again stimulated release of urokinase.

OBJECTIVES

The following experimental objectives are now envisioned for early flights of a space bioreactor.

Conduct cell culture experiments to:

- Study shear regimes that are too low to achieve in 1-g
- Measure the effects of shear forces on a cell membrane and function
- Explore novel culture systems using controlled gas/liquid or liquid/liquid suspensions
- Study transfer of gases to and from the cells in mass culture
- Gain insight into the physical interactions occurring in microgravity among gaseous O₂, liquid media, and CO₂ from cell metabolism

Finally, a space bioreactor can be used to grow and to maintain cells to support development of new cell separation methods and cell biology research in the microgravity environment.

ABSTRACTS

The following abstracts are examples of cell culture studies being developed for flight experiment proposals to be conducted on the Space Shuttle before 1990. Two ground support research projects are also included.

EFFECTS OF PRESSURE AND SHEAR STRESS ON CULTURED CELLS (D. R. Morrison, JSC, R. M. Nerem, Univ. Houston, L. V. McIntire, Rice Univ.)

- Hypothesis

Mechanical stress exerted on cells at less than the force required to rupture the cell membrane will result in changes in cell morphology and membrane mechanical properties. It may cause increased release of product proteins or stress response proteins.

- Rationale/justification

Data from endothelial cell cultures show that prostaglandin secretions can be stimulated to 16 times normal by proper shear stress regimes. Stathopoulos and Hellums reported a relationship between shear force and urokinase release by HEK cells.¹³

- Objectives

- To develop techniques wherein appropriate regulation of shear forces stimulates the release of target products from the cells
- To understand mechanisms of damage to suspension cultures of mammalian cells and to devise improved methods for culture in a stirred suspension reactor

- Proposed experiments

Using the flow chamber described by Stathopoulos and Hellums,¹³ we will run the following studies:

- Flow chamber study of the effects of various fluid shear stresses on cultured monolayer kidney cells
- Studies of changes in morphology and cellular alteration before, during, and after each short or long exposure to a laminar flow field

Under the contract with the Bioprocessing Research Center at UTHSCH, Robert M. Nerem and Murina J. Revesque, University of Houston, have proposed several experiments:

- Determine the effects of pressure and shear stress on cultured kidney cells, including the influence of the degree of confluency, age, passage number, etc.
- Evaluate the effects of different substrates on the tolerance of cultured kidney cells to shear stress and the influence of shear
- Measure the mechanical properties of cultured kidney cells and the change in such properties due to the influence of shear
- Conduct experiments whose purpose will be to determine the behavior of cultured kidney cells on suspended microcarrier beads in the presence of a known shear stress imposed by flow through a parallel plate, channel flow device.

THE EFFECT OF UNIQUE FLUID DYNAMIC PROPERTIES ON CELLS CULTURED IN MICROGRAVITY (D. R. Morrison, JSC)

- Hypothesis

Microgravity will alter the effective mass transfer coefficients for transfer of O_2 (g) into medium and from medium to a cell or enzyme in a slowly stirred suspension culture system.

- Rationale/justification

Fester et al. proposed that certain surface tension forces insignificant in 1-g will alter fluid behavior in space.⁵ Mattoni observed enhanced growth of microorganisms in microgravity.⁹ Nyiri attributed this to better O_2 transfer in the microgravity environment.¹²

- Objective

Measure mixing characteristics and phase-transfer phenomena in a bioreactor equipped with special instrumentation.

- Proposed experiment

Compare the rate of the enzymatic oxidation of glucose to D-glucose- γ -lactone in space and on Earth, proposed by Nyiri¹² (proposal in response to NASA AO-77-3) based on a technique of Weibel and Bright.¹⁶ Preliminary work has already been done by Charles and Nyiri (midterm report of contract NAS 9-15619, 1979).

- Issues

This study may be a necessary part of fine tuning the mixing and oxygenation components of the bioreactor before a cell culture can be adequately sustained under fully optimum conditions in microgravity.

CULTURE OF CELLS IN A CONTROLLED FOAM IN THE ABSENCE OF BUOYANCY (D. R. Morrison, JSC)

- Hypothesis

A steady-state condition can be achieved in a space bioreactor such that a foam can be maintained by introduction of oxygen bubbles and generation of CO_2 . The very high stability of this foam will allow a unique opportunity to culture cells in the liquid phase directly adjacent to air or oxygen bubbles without the usual disadvantage of bubble buoyancy.

- Rationale/justification

In experiments done in Apollo spacecraft, foams were easier to generate and were more stable than on Earth. This hypothesis carries these observations to a novel, practical application.

- Objectives

- To characterize O_2 transport from gas bubbles to cells in the absence of buoyancy
- To find better ways to transport O_2 to living cells in a stirred suspension reactor

- Issues

Oxygenation of cell culture in 1-g is very inefficient because the densities of O_2 (g) and H_2O (l) differ greatly, and because the bubbles of oxygen coalesce readily. Since oxygen has very low solubility in aqueous medium, sparging rates must be high to deliver enough oxygen. High sparging rates, in turn, cause high shear which leads to cell damage.

CULTURE OF CELLS USING FLUOROCARBON OXYGENATORS IN ABSENCE OF DENSITY-DRIVEN SEPARATION (D. R. Morrison, JSC and W. B. Armiger, Bio-Chem Technologies, Inc.)

- Hypothesis

Oxygen and CO_2 are quite soluble in certain fluorocarbon liquids which are very dense (S.G. = 1.7).

It is proposed that oxygen distribution to cell cultures can be enhanced by dissolving it in immiscible fluorocarbons which can be dispersed in the aqueous medium. Since the dispersion will be stable in microgravity, it will be possible to study this novel oxygenation technique properly.

- Rationale/justification

This concept has been tried with terrestrial cultures by Bio-Chem Technologies (Malvern, Pennsylvania). Although density differences caused rapid separation of the two phases, the fluorocarbons dissolve a relatively large amount of O_2 and do provide more O_2 to microbial cells in a suspension type fermentor.

- Objective

To explore better ways to oxygenate cell cultures. To simplify development of practical methods, data from experiments in microgravity may aid implementation of this system in terrestrial cultures. Additional data will, of course, be gained on liquid/liquid interactions as opposed to the liquid/gas interactions in the preceding abstract.

- Issues

- This seems to be an ideal candidate for evaluation in a space bioreactor.
- Need to develop method of dispersion and characterize how long emulsion will remain intact in microgravity.

PHYSIOLOGY OF CELLS CULTURED IN MICROGRAVITY (D. R. Morrison, JSC and R. M. Nerem, Univ. Houston)

- Hypothesis

Cells cultured in the absence of gravity may adapt in ways that are expressed in changes in cell morphology or biochemical functions.

- Rationale/justification

Early experiments with cells growing under weightlessness indicated that the cell density of Salmonella typhimurium was significantly higher than on Earth.⁹ The increase was attributed to enhanced efficiency of nutrient transfer to and waste product removal from the cells. Also cell attachment to microcarriers was significantly increased shortly after being mixed together in microgravity.¹⁵ On the other hand, the study of diploid human embryonic lung cells showed no changes from an Earth control.¹⁰ A long-term study is required to find and characterize any changes in cell structure and function.

- Objective

Measure a variety of morphological and biochemical characteristics of cells on Earth and after acclimation to microgravity. Planned analyses include 2-D electrophoretic mapping of intracellular proteins, cell survival, cell attachment to beads, secretion of natural cell products, total protein concentration, DNA and RNA content, and examination of the cytoskeleton.

- Issue

This may have a bearing on long-term effects of microgravity on humans.

GROUND SUPPORT RESEARCH PROJECTS

ANALYSIS OF THE PROTEINS PRODUCED BY HEK CELLS (D. R. Morrison, JSC and M. Z. Atassi, Baylor College of Medicine)

- Hypothesis

Analytical methods can be devised to separate and quantify a mixture of proteins, which includes several forms of the plasminogen activators and their inhibitors.

- Rationale/justification

Bioassays exist, but these do not measure proenzyme (inactive) nor inhibitors. Specific methods exist for plasminogen activators, but other proteins could escape detection. A means of rapidly assessing the protein production of the bioprocess is desirable.

- Objective

- Develop rapid, general analyses for the high molecular weight fraction that will be the product of the bioreactor. Detect changes in the composition of this fraction.
- Use the bioreactor to provide secreted protein products for purification in space using the McDonnell Douglas CFES apparatus or other NASA sponsored techniques for bioseparation in microgravity.

- Issues

- The proteins involved are roughly similar. Their separation will be a challenge probably requiring CFES, high pressure liquid chromatography (HPLC), or other special techniques.
- A chromatographic method is desirable as an interim step because it allows quantitative analysis of new compounds or loss of old ones as the process changes.
- A radioimmunoassay for urokinase and related compounds is being developed by Dr. Zouhair Atassi, Baylor College of Medicine, under the UTHSC contract NAS9-17403.

- The UTHSC has a task to perfect CFES separation of urokinase from culture medium.
- Methods are needed for the development phase as well as the operational phase.

PROCESS CONTROL SCHEMES FOR A MAMMALIAN CELL CULTURE (C. F. Goochee, Univ. Houston, K. Y. San, Rice University, and D. R. Morrison, JSC)

- Hypothesis

That the requirements for maintenance of these cells are rigid enough to warrant an advanced type of process control.

- Rationale/justification

Existing schemes are not capable of the degree of feedback needed to control a bioreactor through growth and production phases.

- Objective

Automatic control of the bioreactor from inoculation through growth phase into production phase.

- Experiments proposed

Modelling of the process control scheme is being conducted by Dr. Goochee, University of Houston, under the UTHSC contract. The control scheme proposed by the principal investigator has a loop that controls variables such as pH, dissolved oxygen, CO₂, redox potential, and a supervisory loop that detects and connects changes in the metabolic activity of the cell. A fluorescent device to measure cell metabolism by NADH₂ concentration (Bio-Chem Technology, Malvern, Pennsylvania) will be the first element of this loop to be tested by Dr. Morrison at JSC. Dr. Ka-Yiu San, Rice University, also is investigating design aspects of the controller scheme under the UTHSC contract.

- Issues

The monitored parameters and control strategy for a space bioreactor probably applies to ground-based systems as well.

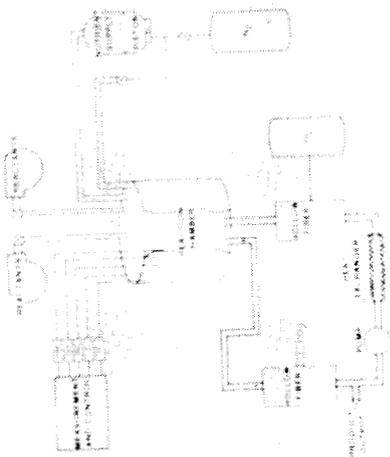
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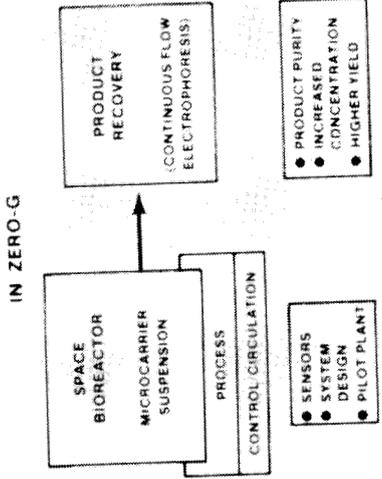
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TABLE 12-1.- UNIVERSITY SUPPORT TASKS FOR BIOREACTOR

<u>TASK</u>	<u>CONTRACTOR</u>
1. ANALYSIS OF CELL CULTURE MEDIUM	BAYLOR COLLEGE OF MEDICINE CELL AND PROTEIN CHEMISTRY LAB (DR. M. Z. ATASSI)
2. SCREENING OF NEW CELL LOTS	PENN STATE UNIVERSITY BIOPHYSICS LAB (DR. P. TODD)
3. FLUID AND GAS DYNAMIC ANALYSIS	U. OF HOUSTON CHEM. ENG. DEPT. (DR. C. GOOCHEE)
4. EFFECTS OF SHEAR ON CELLS AND UK PRODUCTION	RICE UNIV./BAYLOR/IN-HOUSE (DR. KALMAZ - NRC). AND PROCESS CONTROL SYSTEM READY
5. FLUOROMEASURE OF CELL METABOLISM	UNIV. CITY SCI. CENTER (PHIL.) BIOCHEM. TECHNOLOGIES, INC. (DR. BILL ARMIGER)
6. FLUID DYNAMIC STUDIES	U. OF HOUSTON MECH. ENG. DEPT. (DR. R. NEREM)
7. SENSORS, MIXING AND CONTROL SYSTEMS	RICE UNIVERSITY DEPT. CHEM. ENG. (DRS. L. McINTIRE AND T. PAPOUTSAKIS)



- CONCEPTUAL DESIGN OF ZERO-G BIOREACTOR - USES:**
- DEMONSTRATE TECHNOLOGY IMPROVEMENTS BY ELIMINATING SEDIMENTATION OR BUBBLE BOUYANCY
 - CULTURE OF HUMAN CELLS TO OBTAIN HORMONES OR OTHER MEDICAL PRODUCTS
 - TANDEM OPERATIONS WITH CONTINUOUS FLOW ELECTROPHORESIS (CFES)
 - GROWTH/MAINTENANCE OF CELLS FOR SPACE BIOLOGY EXPERIMENTS

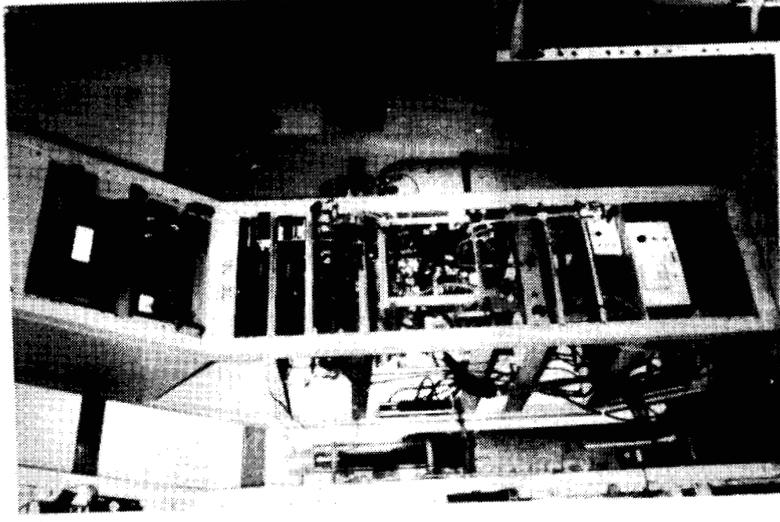


SPACE BIOREACTOR

- SINGLE SPACELAB RACK OR MIDDECK - 3 LOCKER SYSTEM
- SELF CONTAINED DATA RECORDING
- MICROPROCESSOR CONTROLLED OPERATIONS

GROUND BASED PROTOTYPE BIOREACTOR

- SENSOR DEVELOPMENT
- MICROPROCESSOR CONTROL PARAMETERS



BIOREACTOR VERIFICATION TEST UNIT

PROPOSED FOR MIDDECK VERIFICATION TEST IN 1985

- MICROGRAVITY OPERATIONS
- BIOREACTOR CHAMBER
- FLUID CIRCULATION SYSTEM
- SENSOR CONTROL SYSTEM

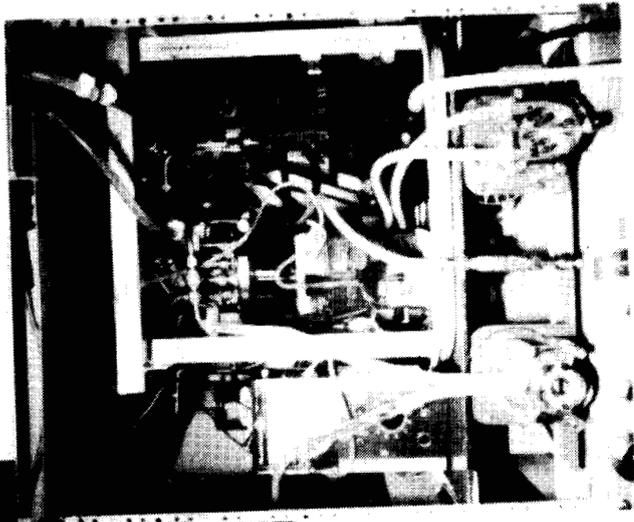


Figure 12-1.- Space bioreactor for cell cultures.

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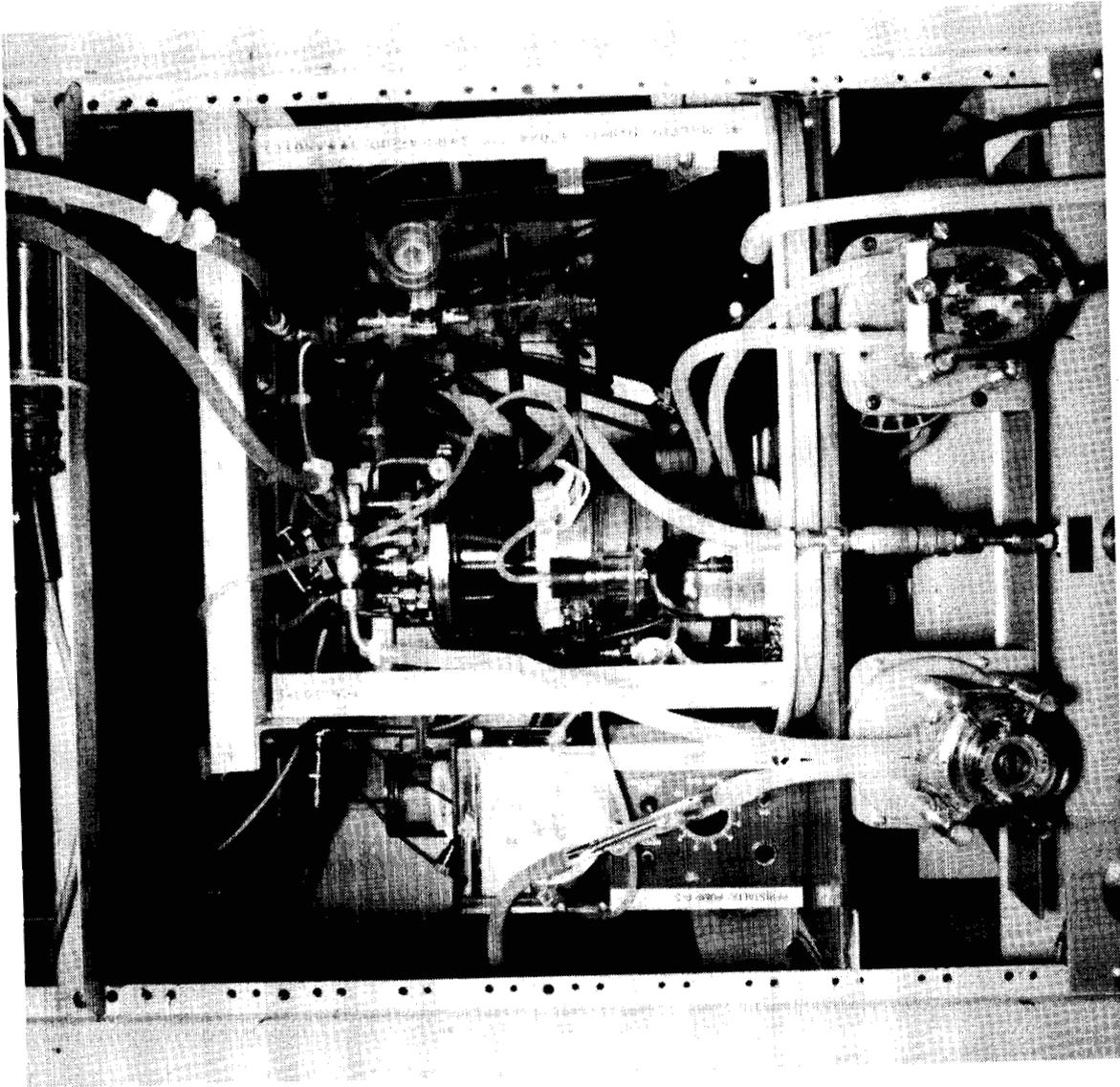


Figure 12-2.- Bioreactor verification test unit.

APPENDIX
LIST OF ATTENDEES

Bruce Edwards Ken Ley, Ph.D.	Lovelace Medical Center
William Armiger, Ph.D.	Bio Chem Technology
Gene Peterson, Ph.D.	Jet Propulsion Laboratory
Giselle Toth, Ph.D.	International Management Consultants
Ki Yu San, Ph.D. Terry Papoutsakis, Ph.D.	Rice University
John Rice, Ph.D. Anthony Dennis, Ph.D.	Battelle Columbus Laboratories
Chris Chow, Ph.D.	3-M Corporation
William Scheid, Ph.D.	Phyto Research, Inc.
Charles Goochee, Ph.D. Robert Nerem, Ph.D.	University of Houston
Leonard Keay, Ph.D.	McDonnell Douglas Astronautics Co.
John Nystrom, Ph.D. William Augerson	Arthur D. Little
Sam Sofer, Ph.D.	University of Oklahoma
Abraham Krikorian, Ph.D.	State University of New York at Stony Brook Technology, Inc.
Andy Anderson William Bowie William Hall Marian Lewis, Ph.D. Kay Elton Jay Cross, Ph.D.	
Errol Kalmaz, Ph.D.	NRC
Bill Huffstetler Mike Richardson	NASA/EX
Ira Wolke	NASA Headquarters
Charles Chassay	NASA/LP
Pete Armitage	NASA/SA
Sam L. Pool, M.D.	NASA/SD
Dennis Morrison, Ph.D. Jerry Carney	NASA/SD3
Dick Williams, Ph.D.	NASA/SN
Robert Rundell	Mississippi State University
Ed Carter D. E. Hawley Irene Verinder	LEMSCO

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15. Supplementary Notes					
16. Abstract Microgravity offers new ways of handling fluids, gases, and growing mammalian cells in efficient suspension cultures. Some conceptual designs for a zero-g fermentor were developed as early as 1969. In 1976 bioreactor engineers designed another system using a cylindrical reactor vessel in which the cells and medium are slowly mixed. The reaction chamber is interchangeable and can be used for several types of cell cultures. NASA has methodically developed unique suspension type cells and recovery apparatus culture systems for bioprocess technology experiments and production of biological products in microgravity. The first space bioreactor has been designed for microprocessor control, no gaseous headspace, circulation and resupply of culture medium, and a slow mixing in very low shear regimes. Various ground-based bioreactors are being used to test reactor vessel design, on-line sensors, effects of shear, nutrient supply, and waste removal from continuous culture of human cells attached to microcarriers. The small (500 ml) bioreactor is being constructed for flight experiments in the Shuttle middeck to verify systems operation under microgravity conditions and to measure the efficiencies of mass transport, gas transfer, oxygen consumption, and control of low shear stress on cells. These proceedings include formal papers which were presented during the Space Bioreactor Science Workshop held at the Johnson Space Center in Houston, Texas, in August 1985. Presentations and discussions included applications of microcarrier cultures, development of the first space bioreactor flight system, shear and mixing effects on cells, process control design considerations, methods to monitor cell metabolism and nutrient requirements during microgravity experiments. Concepts were presented for additional experiments involving culture of plant cells, hybridomas, yeast cells and unique opportunities to study novel methods of culturing fragile human cells in a controlled dispersion of either oxygen bubbles or fluorocarbon (oxygen carrier) in aqueous culture medium.					
17. Key Words (Suggested by Author(s)) bioreactors bioprocessing microgravity applications cells			18. Distribution Statement Unclassified - Unlimited Subject Category 51		
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